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Genetic Expression of Aryl Hydrocarbon Hydroxylase Induction

IV. Interaction of Various Compounds with Different Forms of Cytochrome P-450 and the Effect on Benzo[a]pyrene Metabolism *in Vitro*

F. M. GOUJON, D. W. NEBERT, AND J. E. GIELEN

Section on Developmental Pharmacology, Laboratory of Biomedical Sciences, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20014

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SUMMARY

The phenomena of increased type II binding, measured by pyridine interaction with oxidized cytochrome P-450 *in vitro*, and of decreased type I binding, determined by hexobarbital combination with oxidized P-450 *in vitro*, are related to aryl hydrocarbon hydroxylase induction by aromatic hydrocarbons in genetically responsive mice and do not occur in 3-methylcholanthrene-treated genetically nonresponsive mice. A method for determining specific binding between P-450 and compounds absorbing in the 350–450 nm region is described.

Various lipophilic compounds preferentially inhibit the hydroxylase activity from control or 3-methylcholanthrene-treated genetically nonresponsive mice, whereas other compounds selectively block the 3-methylcholanthrene-induced enzyme activity. By observing the preferential inhibition of one or the other form of hydroxylase activity, one may be able to determine the form of cytochrome P-450 with which a given compound binds. Hence we suggest that α-naphthoflavone, β-naphthoflavone, 2,5-diphenyloxazole, and lindane (γ-hexachlorocyclohexane) interact with a spectrally distinct type *a* species of P-450, the formation of which is associated with hydroxylase induction by aromatic hydrocarbons. Phenylimidazoles, 2-diethylaminoethyl-2,2-diphenyl valerate HCl (SKF 525-A), metyrapone, 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane, 17β-estradiol, Δ^9-tetrahydrocannabinol, cholecalciferol, pyridine, *n*-octylamine, and aniline inhibit aryl hydrocarbon hydroxylase activity by competing with benzo[a]pyrene at a type *b* P-450 active site. A third class of compounds inhibits both type *a* and *b* hydroxylase activities equally, and a fourth class of compounds does not affect either form of the enzyme system. Hexachlorobenzene, α- and β-naphthoflavone, 2,5-diphenyloxazole, lindane, and derivatives of 2-phenylbenzothiazole interact differently with the hepatic enzyme in phenobarbital-treated mice and in control mice. In microsomes from mouse kidney and from rat liver or kidney, the control and 3-methylcholanthrene- or phenobarbital-inducible hydroxylase activities are preferentially inhibited by many of these same compounds in the same manner, indicating that the two forms of the enzyme active site are probably the same in the liver and kidney of the mouse and rat.

INTRODUCTION

At least two types of spectral changes are known (1) to result from substrate interaction with cytochrome P-450 *in vitro*. Whereas treatment with phenobarbital causes increases in both type I and type II

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binding in rat liver microsomes, 3-methylcholanthrene produces a rise only in type II binding (2). α-Naphthoflavone *in vitro* inhibits hepatic aryl hydrocarbon hydroxylase activity from MC[1]-treated rats but not from controls (3). Lipophilic compounds such as MC and its metabolites undoubtedly bind physically and covalently throughout the hepatic lipoidal membranes in the MC-treated animal, thus introducing a possible artifact into studies in which such microsomal membranes are compared with control microsomes (2, 3). Various inbred strains of mice possess aryl hydrocarbon hydroxylase activity inducible by aromatic hydrocarbons, whereas in numerous other strains the enzyme system is not inducible by this class of compounds (4–6). With the use of MC-treated genetically nonresponsive mice, we show here that the increase in type II binding, the decrease in type I binding, and the difference in inhibition by α-naphthoflavone are directly related to the hydroxylase induction process and are not nonspecific effects of polycyclic hydrocarbons bound to the microsomes.

In the preceding paper (6) an association between aryl hydrocarbon hydroxylase induction by various aromatic hydrocarbons and the conversion of hepatic type *b* to type *a* cytochrome P-450 was shown to occur in mice heterozygous or homozygous for the *Ah* allele.[2] Because neither the monoxygenase induction by polycyclic hydrocarbons nor the change in type of P-450 occurs in MC-treated *ahah* mice, we conclude that this conversion of type *b* to type *a* P-450 is linked genetically with the induction process and that the experimental technique for determining the two forms of P-450 by *n*-octylamine binding (8) is not affected simply by MC treatment of the animal. The appearance of a spectrally distinct CO-binding pigment (4, 5, 9, 10), the conversion of type *b* to type *a* P-450 (6, 11), and the increase in one of two high-spin forms of P-450 detectable by EPR spectroscopy at temperatures below 10°K (12) occur in the MC-treated genetically responsive mouse. These findings suggest that aryl hydrocarbon hydroxylase activity induced by aromatic hydrocarbons represents P-450 iron in a preferred high-spin configuration. Moreover, because α-naphthoflavone *in vitro* inhibits hepatic aryl hydrocarbon hydroxylase activity from MC-treated rats but not from controls (3), one interpretation is that the control enzyme activity represents type *b* P-450 and the MC-induced enzyme system reflects type *a* P-450. By comparing the pattern of inhibition by various compounds of aryl hydrocarbon hydroxylase activity from control, MC-nonresponsive, MC-responsive, and PB-treated mice, we provide in this report evidence which strengthens this hypothesis.

[1] The abbreviations used are: MC, 3-methylcholanthrene; PB, phenobarbital; DDT, 2,2-bis-(*p*-chlorophenyl)-1,1,1-trichloroethane.

[2] See "Note Added in Proof" in accompanying paper (6) and *cf. ref.* 7 for review.

MATERIALS AND METHODS

Benzo[*a*]pyrene from Sigma, 7,12-dimethylbenz[*a*]anthracene from Eastman Organic Chemicals, and MC from J. T. Baker Chemical Company were purified by recrystallization from benzene. Sodium phenobarbital was purchased from Merck & Company. From Aldrich Chemical Company we obtained α-naphthoflavone, β-naphthoflavone, DDT, *n*-octylamine, and spectral grade dimethylsulfoxide. Norepinephrine, isoproterenol, 17β-estradiol, dexamethasone, cholesterol, cholecalciferol, sodium laurate, and spironolactone were obtained from Sigma Chemical Company. Testosterone propionate was purchased from Mann Research Laboratories; 2,5-diphenyloxazole, from New England Nuclear; bovine serum albumin, from Armour Pharmaceutical Company; pyridine and aniline, from J. T. Baker Chemical Company; *n*-hexane and cyclohexane, from Eastman Organic Chemicals; 1-butanol, from Allied Chemical Company; 2-chloro-10-(3-dimethylaminopropyl)phenothiazine HCl (chlorpromazine), from Smith Kline & French Laboratories; and perfluoro-*n*-hexane, from Pierce Chemical Company. The remainder of the compounds used in this study were generous gifts: metyrapone (2-methyl-1,2,3,3-pyridyl-1-propanone), from Dr. J. J. Chart, Ciba Pharmaceutical Company; all of the 2-phenylbenzothiazole derivatives, from Dr. J. L. Leong, University of Minne-

sota Medical School; and hexachlorobenzene, γ-hexachlorocyclohexane (lindane), piperonyl butoxide, allylisopropylacetamide, and aminopyrine, from Dr. A. P. Poland, University of Rochester Medical Center. The remaining compounds were generous gifts of scientists at the National Institutes of Health: SKF 525-A, from Dr. J. H. Weisburger; 1-(2-isopropylphenyl)imidazole and 1-(2-cyanophenyl)imidazole, from Dr. J. W. Daly; sodium hexobarbital, from Dr. F. J. Wiebel; Δ^9-tetrahydrocannabinol, from Dr. L. Lemberger; hemin, from Dr. A. T. Ness; and diethylstilbestrol, from Dr. P. Kohler.

Treatment of the mice, preparation of microsomes, and assay of aryl hydrocarbon hydroxylase activity were performed as described in the preceding paper (6). MC-treated mice received 80 mg of MC in corn oil per kilogram of body weight intraperitoneally 24 hr before death; controls received corn oil only. PB-treated mice received 80 mg of PB in 0.90% NaCl per kilogram of body weight intraperitoneally on each of 3 successive days before death.

Spectrophotometry. Difference spectra that result when hexobarbital or pyridine binds to microsomal hemoprotein suspensions were determined by the method of Remmer *et al.* (13) with the use of a Shimadzu model MPS-50L multipurpose recording spectrophotometer. Cytochrome P-450 measurements were carried out as discussed in the preceding paper (6). A technique for determining type I or type II spectral binding for colored chemicals was performed with the use of tandem cuvettes having dual compartments measuring 4.7 mm each (Hellma Cells, Jamaica, N. Y.). In the compartment closer to the photomultiplier tube was placed the microsomal suspension containing 2–6 mg of protein per milliliter; the compartment farther from the photomultiplier tube contained 25 mg of albumin per milliliter of 30% glycerol–0.25 M potassium phosphate buffer, pH 7.25. After a balanced baseline had been established, the test compound was added in 10 μl of solvent to the microsomes of the experimental cuvette and to the albumin solution of the reference cuvette; 10 μl of solvent alone were added to the microsomes of the reference cuvette and to the albumin solution of the experimental cuvette. Reproducible type I or type II spectra could thus be obtained by this method while canceling undesirable interfering absorption in the 350–450 nm region.

Effects of test compounds on benzo[α]pyrene hydroxylation in vitro. The effects of various test compounds at several concentrations on aryl hydrocarbon hydroxylase activity from control, MC-treated genetically nonresponsive, MC-treated genetically responsive, and PB-treated mice were examined. To achieve the final concentrations depicted in the figures, the compounds were added in 10 μl of solvent to 0.95 ml of the reaction mixture (11) containing 50 μmoles of potassium phosphate buffer (pH 7.25), 0.36 μmole of NADPH, 0.39 μmole of NADH, 600 μg of bovine serum albumin, 3 μmoles of $MgCl_2$, and 0.4–1.0 mg of the hepatic microsomes. The best solvent (methanol, ethanol, acetone, ether, dimethylsulfoxide, or water) was chosen for each compound, and 10 μl of this solvent in each of four separate flasks per experiment were used for determining the 100% level of aryl hydrocarbon hydroxylase activity in the absence of any inhibitor. None of the solvents inhibited the enzyme activity more than 15%. The reaction mixture was then incubated at 37° for 1 min to allow adequate binding of the test compound to the microsomal membranes. Then 80 nmoles of the substrate benzo[a]pyrene in 40 μl of methanol were added to each flask, and the amount of 3-hydroxybenzo[a]pyrene formed in 10 min at 37° was determined (11). With hemin (not shown), but not with any other of the more than 40 compounds tested, the extraction of 3-hydroxybenzo[a]pyrene into alkali was affected, so that the enzyme activity could not be accurately determined in the presence of hemin in the reaction mixture. The procedure for kidney microsomes was the same as that for hepatic microsomes, except that the pH of the reaction mixture was 7.5 and the 37° incubation with the substrate benzo[a]pyrene was carried out for 30 min instead of 10 min (11). One unit of aryl hydrocarbon hydroxylase activity is defined (4–6, 10, 11) as that amount of enzyme catalyzing per minute at 37° the formation of hydroxylated prod-

uct causing fluorescence equivalent to that of 1 pmole of 3-hydroxybenzo[a]pyrene.

RESULTS

Use of dual-compartment cuvettes for determining type I or type II spectra of colored compounds. The top of Fig. 1 illustrates a typical spectrum obtained when benzo[a]-pyrene was mixed with microsomes in the experimental cuvette and solvent alone was added to microsomes in the reference cuvette. If albumin was used instead of hepatic microsomes, we found the same spectrum with minor shifts in the spectral maxima and minima; results similar to those with benzo[a]pyrene were also observed with 7,12-dimethylbenz[a]anthracene, MC, and benz[a]anthracene (not shown). These findings are in accord with a previous report (14) in which hepatic microsomes from benzo[a]pyrene-treated rats or MC-treated rabbits appeared spectrally similar to control rat or rabbit microsomes to which the polycyclic hydrocarbon had been added

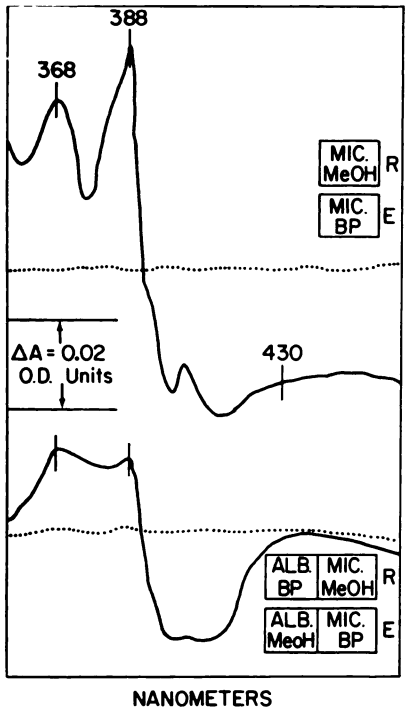

FIG. 1. *Difference spectra illustrating nonspecific binding of benzo[a]pyrene (BP) to mouse liver microsomes (upper) and specific binding of benzo[a]pyrene to hepatic microsomal P-450 (lower)*

The dotted line in each case represents the balanced baseline with suspensions of oxidized microsomes in both reference (R) and experimental (E) cuvettes. The upper solid line indicates the difference in optical density when benzo[a]-pyrene was added in 10 μl of methanol (MeOH) to the microsomes (MIC.) in the experimental cuvette and 10 μl of methanol alone were added to the microsomes in the reference cuvette. The lower solid-line spectrum represents the difference in absorbance when benzo[a]pyrene was mixed with liver microsomes in the experimental tandem cuvette and with albumin (ALB.) in the reference tandem cuvette, as shown in the key at lower right and described under MATERIALS AND METHODS.

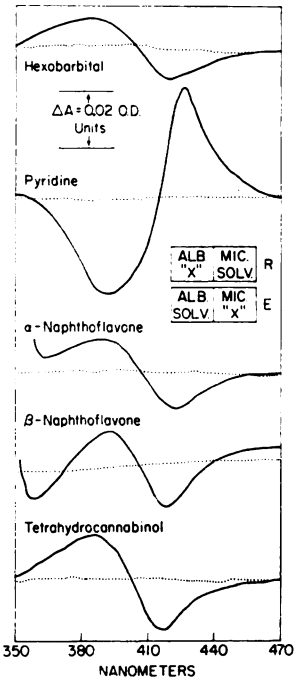

FIG. 2. *Difference spectra showing specific binding of various compounds to hepatic microsomal P-450.*

In each instance the technique described under MATERIALS AND METHODS and shown diagrammatically in the key at right was employed. After a balanced baseline had been obtained, the compound to be examined ("X") was added to microsomes (MIC.) in the experimental cuvette (E) and to albumin (ALB.) in the reference cuvette (R); an equal amount of solvent alone (SOLV.) was then added to the microsomes in the reference cuvette and to the albumin in the experimental cuvette.

in vitro. Thus the light yellow color of aromatic hydrocarbons interferes in the 350–450 nm region, thereby making difficult any determination whether the compound is a type I or type II (1, 13) substrate of P-450. This difficulty was circumvented with the use of dual-compartment tandem cuvettes, as shown in the bottom of Fig. 1. In the difference spectrum between benzo[*a*]pyrene bound to hepatic microsomes in the experimental tandem cuvette and benzo[*a*]pyrene bound to albumin in the reference tandem cuvette, the absorption caused by the polycyclic hydrocarbon nonspecifically bound to protein is canceled. The remaining optical differences clearly indicate that the specific binding of benzo[*a*]pyrene to microsomal P-450 represents a type I interaction. The same conclusion was reached for 7,12-dimethylbenz[*a*]anthracene, MC, and benz[*a*]anthracene.

Figure 2 shows that, with the use of these same dual-compartment cuvettes, hexobarbital and pyridine elicited the expected type I and type II spectra, respectively. Although α-naphthoflavone, β-naphthoflavone, and Δ^9-tetrahydrocannabinol produced interfering absorbance below 420 nm when examined by the usual method (13), typical type I spectra were observed with the tandem cuvette technique. That Δ^9-tetrahydrocannabinol is a type I compound suggests that it is metabolized by a hepatic P-450-mediated monooxygenase. The 11-hydroxy metabolite rather than the parent compound has been recently implicated (15) in the hallucinogenic effect of the drug.

Genetically determined changes in type I and type II binding after MC or PB treatment of mice. Table 1 summarizes the magnitude of type I or type II binding relative to microsomal protein or P-450 content in control, MC-treated, and PB-treated *AhAh* or *ahah* mice. Whereas MC treatment did not significantly ($p > 0.05$) change the amount of hexobarbital binding per milligram of microsomal protein or the amount of type II binding relative to total P-450 content, the quantity of type I binding relative to P-450 concentration was decreased about 40%, and the amount of pyridine binding per milligram of microsomal protein was increased about 40%, in MC-treated *AhAh* mice, but remained un-

TABLE 1

Relationship between hexobarbital and pyridine binding to hepatic cytochrome P-450 in different strains of inbred mice

Treatment of the mice and preparation of liver microsomes are described in MATERIALS AND METHODS of the preceding paper (6). Difference spectra were obtained with final concentrations of 5 mM hexobarbital or 30 mM pyridine in the microsomal suspension in the experimental cuvette (1, 12). The peak to trough differences are expressed relative to microsomal protein and cytochrome P-450 concentrations. These values represent the mean ± standard deviation of five such determinations on microsomes pooled from two mice each time. The results from C57BL/6N, C3H/HEN, BALB/cAnN, and AL/N strains, in which aryl hydrocarbon hydroxylase is inducible, were relatively similar and are averaged as the *AhAh* group; the results from DBA/2N, NZW/BLN, and NZB/BLN strains, in which the enzyme system is not inducible by aromatic hydrocarbons, were also relatively similar and are averaged as the *ahah* group.

Proposed genotype at *ah* locus	Previous treatment	Hexobarbital binding		Pyridine binding	
		$\Delta A_{386-419}$/mg microsomal protein	$\Delta A_{386-419}:\Delta A_{450-490}$	$\Delta A_{426-390}$/mg microsomal protein	$\Delta A_{426-390}:\Delta A_{450-490}$
AhAh	None	0.0076 ± 0.004	0.14 ± 0.02	0.029 ± 0.002	0.59 ± 0.03
ahah		0.0100 ± 0.002	0.17 ± 0.03	0.028 ± 0.004	0.52 ± 0.02
AhAh	MC	0.0071 ± 0.004	0.08 ± 0.02	0.042 ± 0.006	0.58 ± 0.03
ahah		0.0100 ± 0.004	0.18 ± 0.03	0.027 ± 0.004	0.53 ± 0.02
AhAh	PB	0.036 ± 0.005	0.19 ± 0.02	0.062 ± 0.004	0.43 ± 0.02
ahah		0.027 ± 0.006	0.19 ± 0.03	0.058 ± 0.003	0.41 ± 0.03

altered in MC-treated *ahah* mice or in control mice. We found that similar magnitudes of decrease in hexobarbital binding and of increase in pyridine binding occurred in each of four inbred strains having the MC-inducible hepatic aryl hydrocarbon hydroxylase activity and that these changes did not occur in three MC-treated genetically nonresponsive strains. In a single experiment this same increase in type II and decrease in type I binding was found with liver microsomes from MC-treated *Ahah* (B6D2 F_1 heterozygote) mice. Thus these changes in hexobarbital or pyridine binding are unrelated to bound inducer or metabolite *in vivo*, as suggested by Schenkman (16), and we suggest that these changes are related to our observation (6) that aryl hydrocarbon hydroxylase induction by aromatic hydrocarbons in genetically responsive mice involves an increase in type *a* P-450.

Of further interest was the shape of the pyridine-produced type II spectrum after treatment with MC: for each *AhAh* mouse the trough had a sharp minimum at about 392 nm, whereas for each *ahah* mouse the trough was flat between 392 and 410 nm. In fact, the curves were remarkably similar to the *n*-octylamine difference spectra shown in Fig. 1 of the preceding paper (6). The difference spectra for basic amines bound to hepatic P-450 from adult male control and benzo[*a*]pyrene-treated rats (16) are similar in shape to those from control and MC-treated *AhAh* mice, respectively.

As expected, PB administration to either *AhAh* or *ahah* mice caused similar increases in both type I and type II spectra. These 2- to more than 4-fold increases in both types of difference spectra are similar to those (2) for liver microsomes from PB-treated rats.

Inhibition or stimulation by test compounds in vitro of enzyme activity from control, MC-treated, or PB-treated mice. The remaining figures in this report illustrate the effects of numerous lipophilic compounds on the aryl hydrocarbon hydroxylase system from control, MC-nonresponsive, MC-responsive, and PB-treated mice. In Figs. 3–6 only inbred C57BL/6N and DBA/2N mice were used. We hoped to answer several questions by carrying out such a survey. First, could we distinguish different forms of the monooxygenase activity that reflect type *a* or type *b* P-450? Second, could lipophilic compounds which are known to interact with microsomal membranes be classified as to their effect on type *a* or type *b* hydroxylase activity? Third, are these distinguishable effects on the hepatic aryl hydrocarbon hydroxylase activity by these different compounds the same in nonhepatic microsomes?

Figure 3 shows that, in the presence of eight different substrates or inhibitors *in vitro*, hepatic aryl hydrocarbon hydroxylase activity in MC-treated genetically responsive mice is distinctly different from that in control, MC-treated DBA/2N, or PB-treated mice. For example, 500 μM α-naphthoflavone blocked more than 80% of the MC-induced hydroxylase activity, whereas the enzyme system from control or MC-treated DBA/2N mice was unaffected. The effects of β-naphthoflavone and lindane were very similar to that of α-naphthoflavone, while the effect of spironolactone was less pronounced. The effects of 1-(2-isopropylphenyl)imidazole, SKF 525-A, metyrapone, and DDT were just the opposite, since these compounds more strongly inhibited the aryl hydrocarbon hydroxylase activity from control, MC-nonresponsive, or PB-treated mice, compared with effects on the MC-induced enzyme system. For example, 50 μM 1-(2-isopropylphenyl)imidazole blocked about 90% of the enzyme activity from control, MC-treated DBA/2N, or PB-treated mice, but only about 30% of the MC-induced enzyme system. According to our proposed scheme in Fig. 3 of the preceding paper (6), MC treatment evokes an increase in type *a** P-450, presumably related to the spectral blue shift in the Soret peak of the reduced P-450–CO complex and to the increase in one of two high-spin P-450 species detectable by EPR (12). Hence we suggest that α-naphthoflavone, β-naphthoflavone, and lindane interact with this new form of P-450, thereby competing with benzo[*a*]pyrene for the *a** P-450 active site; likewise, 1-(2-isopropylphenyl)imidazole, SKF 525-A, metyrapone, and DDT bind to type *b* P-450, competing with benzo[*a*]pyrene for the *b** P-450 enzyme site. This interpretation for metyrapone is in agreement with recent report (17) describing the competitive

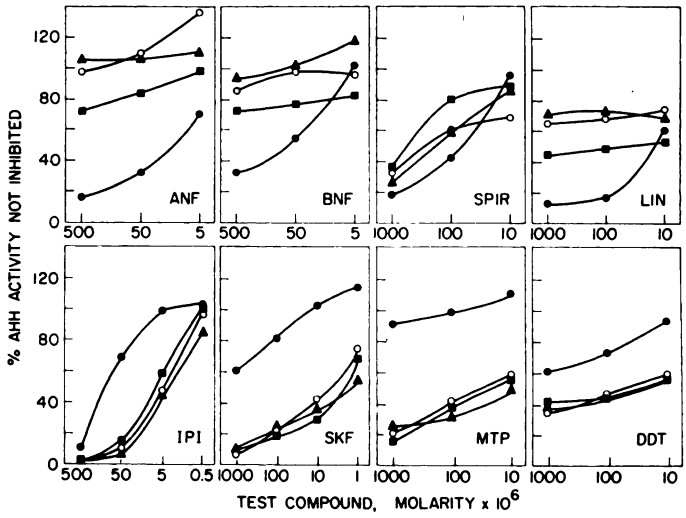

FIG. 3. *Effects of various test compounds on hepatic aryl hydrocarbon hydroxylase (AHH) activity in vitro from control* (○), *MC-treated genetically nonresponsive DBA/2N* (▲), *MC-treated genetically responsive C57BL/6N* (●), *or PB-treated* (■) *mice*

In no instance was the effect of a test compound on aryl hydrocarbon hydroxylase activity different between control C57BL/6N and DBA/2N mice or between PB-treated C57BL/6N and DBA/2N mice. Each point represents duplicate determinations carried out two to five times until reproducibility was satisfactory. Differences of more than 20% in the inhibition of enzyme activity are statistically ($p < 0.05$) significant. ANF, α-naphthoflavone; BNF, β-naphthoflavone; SPIR, spironolactone; LIN, lindane; IPI, 1-(2-isopropylphenyl)imidazole; SKF, SKF 525-A; MTP, metyrapone; DDT, *p,p'*-DDT. The specific hepatic hydroxylase activity averaged about 500 for the control and MC-treated DBA/2N mice, about 2500 for the MC-treated C57BL/6N mice, and about 1000 for the PB-treated mice (6). Thus, for example, 40% of the "aryl hydrocarbon hydroxylase activity not inhibited" represents specific activities of about 200, 1000, and 400 for the three groups, respectively. These same values for hepatic hydroxylase activities also hold true for Figs. 4–6.

inhibition of camphor metabolism by metyrapone interaction with low-spin P-450. Using both *a** and *b** inhibitors, we constructed velocity-substrate curves and Lineweaver-Burk plots for the various aryl hydrocarbon hydroxylase activities, and in each case a competitive type of inhibition was most closely approximated (18).[3]

A further noteworthy observation in Fig. 3 is that the hydroxylase activity in PB-treated mice was more inhibited by α-naphthoflavone, β-naphthoflavone, and lindane than were the control or MC-noninducible enzyme systems. This effect was not seen with the "*b** inhibitors" at the bottom of Fig. 3. These observations indicate that detectable differences exist between hepatic microsomes from control or MC-treated DBA/2N mice and microsomes from PB-treated mice, and that if α-naphthoflavone, β-naphthoflavone, and lindane specifically interact with type *a** P-450, then microsomes from PB-treated animals have measurably more high-spin aryl hydrocarbon hydroxylase activity than do membranes from control or MC-treated *ahah* mice. This hypothesis is in fact supported by recent EPR data (12). In rat liver, PB administration also has been reported to cause differences—similar to those produced by MC although to a lesser degree—in tritium exchange with acetanilide as a substrate (19) and in ethylisocyanide difference spectra (20).

Figure 4 shows the effects of four 2-phenylbenzothiazole derivatives on the various aryl hydrocarbon hydroxylase systems. The apparent "stimulatory" effect of 2-(4'-

[3] F. M. Goujon, J. E. Gielen, and D. W. Nebert, unpublished observations.

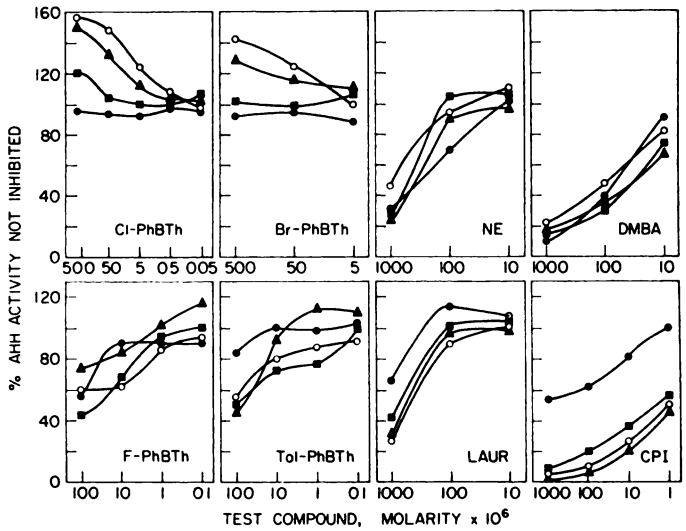

FIG. 4. *Effects of various test compounds on hepatic aryl hydrocarbon hydroxylase (AHH) activity in vitro from control* (○), *MC-treated DBA/2N* (▲), *MC-treated genetically responsive C57BL/6N* (●), *or PB-treated* (■) *mice*

Cl-PhBTh, 2-(4′-chlorophenyl)benzothiazole; Br-PhBTh, 2-(4′-bromophenyl)benzothiazole; NE, norepinephrine; DMBA, 7,12-dimethylbenz[*a*]anthracene; F-PhBTh, 2-(4′-formylphenyl)benzothiazole; Tol-PhBTh, 2-*p*-toluenesulfonamidobenzothiazole; LAUR, sodium laurate; CPI, 1-(2′-cyanophenyl)-imidazole.

chlorophenyl)benzothiazole and 2-(4′-bromophenyl)benzothiazole on enzyme activity, especially from control or MC-treated DBA/2N mice, was different from the slight inhibition of each enzyme system by 2-(4′-formylphenyl)benzothiazole and 2-*p*-toluenesulfonamidobenzothiazole. These two halogenated derivatives induce hepatic and nonhepatic aryl hydrocarbon hydroxylase activity in *AhAh* or *Ahah* but not in *ahah* mice, whereas the latter two derivatives are ineffective as inducers *in vivo* (6).[3] Norepinephrine and isoproterenol (the latter not shown) similarly inhibited both types of hydroxylase activity only at 1 mM concentrations. As expected, 7,12-dimethylbenz[*a*]anthracene blocked both forms of the enzyme equally. Inhibition of 50% was found at approximately 80 μM 7,12-dimethylbenz[*a*]anthracene; since the concentration of the substrate benzo[*a*]pyrene was 80 μM in the reaction mixture, this indicates that both polycyclic hydrocarbons probably have similar affinities for both *b** and *a** aryl hydrocarbon hydroxylase active sites. Chlorpromazine (not shown) also inhibited both forms of hydroxylase activity equally. Laurate was inhibitory only at 1 mM levels, and the effect on *a** activity was slightly less than that on *b** enzyme activity. The preferential inhibition of *b** activity by 1-(2-cyanophenyl)-imidazole was very similar to that displayed by the isopropyl derivative in Fig. 3.

Figure 5 shows that PB *in vitro* was not inhibitory to either form of aryl hydrocarbon hydroxylase activity, even at more than 10 times the concentration of the substrate benzo[*a*]pyrene. The response to hexobarbital was very similar to that shown for laurate in Fig. 4. The inhibition of *a** hydroxylase activity and the apparent stimulation of *b** activity at 10 μM concentrations of aminopyrine and allylisopropylacetamide were identical with each other but are not understood. Preferential inhibition of type *b** hydroxylase activity by Δ^9-tetrahydrocannabinol, *n*-octylamine, piperonyl butoxide, pyridine, and aniline (the last not shown) was witnessed.

In Fig. 6 the inhibitory profile of hepatic aryl hydrocarbon hydroxylase activities by 2,5-diphenyloxazole was similar to that observed with the flavones and lindane (Fig. 3), except that the PB-induced enzyme

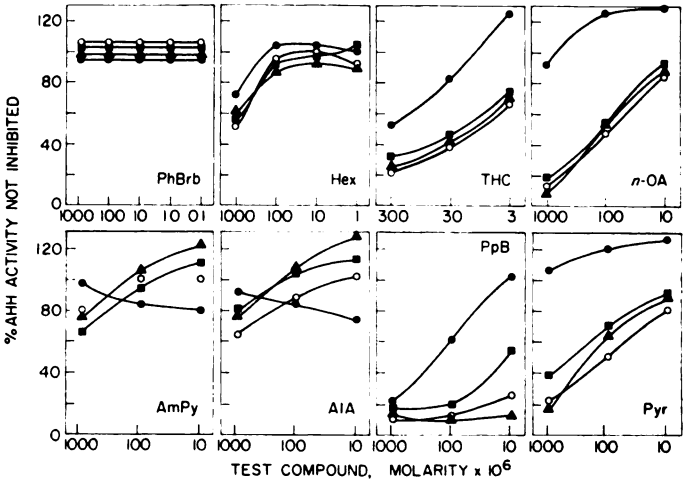

FIG. 5. *Effects of various test compounds on hepatic aryl hydrocarbon hydroxylase (AHH) activity in vitro from control* (○), *MC-treated DBA/2N* (▲), *MC-treated genetically responsive C57BL/6N* (●), *or PB-treated* (■) *mice*

PhBrb, phenobarbital; Hex, sodium hexobarbital; THC, Δ^9-tetrahydrocannabinol; *n*-OA, *n*-octylamine; AmPy, aminopyrine; AIA, allylisopropylacetamide; PpB, piperonyl butoxide; Pyr, pyridine.

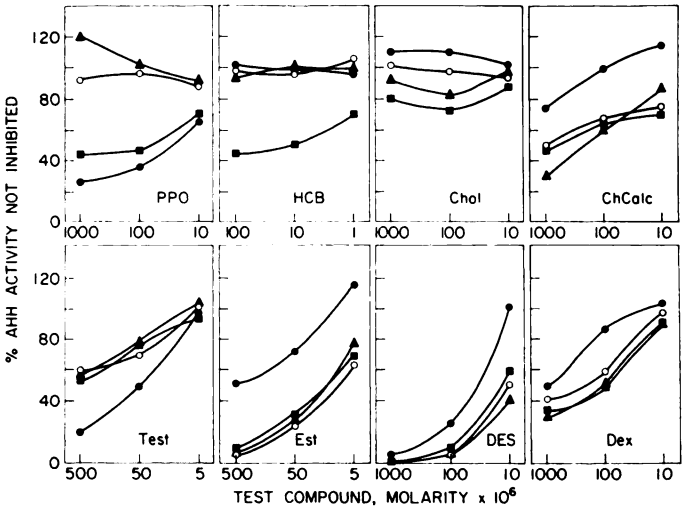

FIG. 6. *Effects of various test compounds on hepatic aryl hydrocarbon hydroxylase (AHH) activity in vitro from control* (○), *MC-treated DBA/2N* (▲), *MC-treated genetically responsive C57BL/6N* (●), *or PB-treated* (■) *mice*

PPO, 2,5-diphenyloxazole; HCB, hexachlorobenzene; Chol, cholesterol; ChCalc, cholecalciferol; Test, testosterone; Est, 17β-estradiol; DES, diethylstilbestrol; Dex, dexamethasone.

system was inhibited to almost the same extent as the MC-induced enzyme. Significant enhancement of the control and MC-noninducible enzyme activities by 1 mM 2,5-diphenyloxazole was also observed in some experiments with high concentrations of α- or β-naphthoflavone. Hexachlorobenzene produced a unique response: 50% of the PB-induced activity was inhibited by 10 μM hexachlorobenzene, while no inhibition of the control, MC-noninducible, or MC-induced enzyme was seen at 100 μM amounts of this

substance. Hexachlorobenzene apparently is not metabolized at all by P-450 (21); if this property is related to the unusual inhibitory profile caused by this compound, the relationship is unclear. Cholesterol even at 1 mM was not significantly inhibitory to the hydroxylase systems; we found a similar lack of inhibition with cyclohexane, *n*-hexane, perfluoro-*n*-hexane, and 1-butanol, each at concentrations up to 1 mM (not shown). Cholecalciferol, on the other hand, inhibited *b** significantly more than *a** hydroxylase activity. Of the four steroids or analogues tested, testosterone preferentially inhibited *a** aryl hydrocarbon hydroxylase activity, whereas diethylstilbestrol and dexamethasone inhibited *b** activity to a slightly greater extent. However, 17β-estradiol is clearly a "*b** inhibitor." These findings are consistent with our earlier observation (22) that competitive inhibition of benzo[*a*]pyrene hydroxylation by 17β-estradiol *in vitro* was more marked than that by testosterone. The rather strong inhibition of a hepatic monooxygenase activity by diethylstilbestrol suggests that the stilbene nucleus interacts with hepatic cytochrome P-450. This fact may be of interest in view of the recently implicated association (23) between diethylstilbestrol therapy of mothers during pregnancy and vaginal adenocarcinoma in the offspring at puberty.

Figure 7 shows the inhibition or apparent stimulation of renal aryl hydrocarbon hydroxylase activities by eight test compounds that had been also tested on the hepatic hydroxylase systems. The type of response in each instance was the same as had been found with the liver hydroxylases, with the possible exception of laurate. Hence α-naphthoflavone, 2,5-diphenyloxazole, and spironolactone (to a lesser degree) preferentially inhibited the MC-induced enzyme activity; 1-(2-isopropylphenyl)imidazole, SKF 525-A, and metyrapone preferentially blocked the control or MC-noninducible enzyme system; and 500 μM 2-(4′-chlorophenyl)benzothiazole significantly enhanced the hydroxylase activities. A slightly preferred inhibition of the MC-induced activity by laurate was seen,

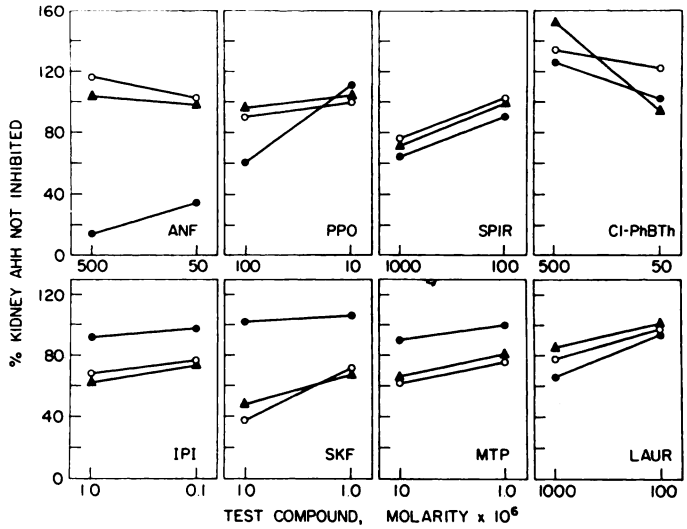

FIG. 7. *Effects of various test compounds on kidney microsomal aryl hydrocarbon hydroxylase (AHH) activity in vitro from control* (○), *MC-treated DBA/2N* (▲), *or MC-treated genetically responsive C57BL/6N* (●) *mice*

ANF, α-naphthoflavone; PPO, 2,5-diphenyloxazole; SPIR, spironolactone; Cl-PhBTh, 2-(4′-chlorophenyl)benzothiazole; IPI, 1-(2-isopropylphenyl)imidazole; SKF, SKF 525-A; MTP, metyrapone; LAUR, sodium laurate. The specific aryl hydrocarbon hydroxylase activity averaged about 1.0 for the MC-treated DBA/2N and control mice, and about 25 for the MC-treated C57BL/6N mice (6). Thus, for example, 40% of the kidney "aryl hydrocarbon hydroxylase activity not inhibited" represents specific activities of about 0.4 and 10 for the two groups, respectively.

in contrast to the effect with the liver enzyme (Fig. 4). However, at 1 mM, laurate has solubilizing properties similar to those of a detergent, and we may have observed such physical effects rather than specific chemical effects of the fatty acid.

The same preferential inhibitory or stabilizing effects of α-naphthoflavone, 2,5-diphenyloxazole, 2-(4′-chlorophenyl)benzothiazole, 1-(2-isopropylphenyl)imidazole, metyrapone, 17β-estradiol, and hexachlorobenzene were seen with a second genetically responsive inbred mouse strain (C3H/HEN), a second genetically nonresponsive inbred strain (NZW/BLN), and heterozygote (B6D2 F_1) mice. Very similar effects were also found with control, MC-treated, or PB-treated weanling male Osborn-Mendel rats. Two exceptions were observed: SKF 525-A did not preferentially inhibit the control rat kidney microsomal aryl hydrocarbon hydroxylase activity, and 2-(4′-chlorophenyl)benzothiazole did not consistently enhance the enzyme activity in rat liver, as had been seen with mouse liver.

DISCUSSION

We conclude from these data that there exist at least two forms of aryl hydrocarbon hydroxylase activity, which possibly (6) reflect high- and low-spin P-450, and that the two forms of kidney hydroxylase activity appear identical with those found in liver microsomes. It is therefore likely that an increase in *a** P-450 occurs in kidney as it does in liver microsomes, a possibility raised in the discussion of the preceding paper (6). Moreover, since the enzyme in the MC-treated genetically nonresponsive mouse interacts with numerous lipophilic compounds in the same manner as the control enzyme does, MC administration *per se* does not change the microenvironment of P-450. This finding is in accord with data of the preceding paper, wherein we found (6) that MC treatment of *ahah* mice did not noticeably alter the type of *n*-octylamine binding to P-450. Therefore newly synthesized, induction-specific protein interacts with the microsomal membrane in such a manner that the ligand field of P-450 is physically altered in the *AhAh* or *Ahah* but not in the *ahah* mouse. This genetically linked change in the membrane configuration is thus reflected as a preferred high-spin state of P-450 (i.e., $g = 8.0$) and as marked differences in the effects of various compounds on the different aryl hydrocarbon hydroxylase activities. Finally, some differences are detectable between the interaction of certain compounds with the hepatic aryl hydrocarbon hydroxylase system in PB-treated mice and in control mice.

In Table 1, if the amount of type I binding is divided by the amount of type II binding for each group of mice, the relative changes are similar to the decrease in the type *b*:*a* ratio associated with aryl hydrocarbon hydroxylase induction by aromatic hydrocarbons (6). Metyrapone produces a marked increase in type II binding when *Pseudomonas putida* P-450 is in the high-spin form (17); this increase may represent the larger trough in the 392 nm region. Therefore a relationship may exist among the shape of the type II spectrum caused by pyridine (16) or *n*-octylamine (8) binding to P-450 *in vitro*, the 455 nm peak in the reduced P-450–ethylisocyanide complex (20), and the increase (12) in high-spin P-450 distinguishable by EPR.

It is important to keep in mind that the interaction of a type I substrate with oxidized P-450 *in vitro* is correlated with a conversion of hemoprotein iron from the low-spin to the high-spin state and that the interaction of a type II substrate with the oxidized cytochrome *in vitro* is associated with a conversion of high-spin to low-spin iron (24–26). These facts are not to be confused with our finding (6, 11, 12) that MC administration *in vivo* produces a genetically determined preference of P-450 in the high-spin form. It has been emphasized (27) that the sequential bindings of the two forms of P-450 by *n*-octylamine may correspond to distinct membrane proteins or configurations and not merely to two forms of the hemoprotein in an equilibrium affected by differences in ionic strength or pH.

The preferential effects of numerous compounds on one or the other form of aryl hydrocarbon hydroxylase activity allow us to classify some as either substrates or in-

TABLE 2

Preferential inhibitory effects of various test compounds in vitro on benzo[a]pyrene hydroxylation representing type b or type a* P-450*

Treatment of the mice and preparation of liver microsomes are described in MATERIALS AND METHODS of the preceding paper (6), and the conditions for studying inhibition of benzo[a]pyrene hydroxylation *in vitro* are described in MATERIALS AND METHODS and Fig. 3 of this report. As we have postulated, the *b** column represents aryl hydrocarbon hydroxylase activity involving one form of P-450 from control or MC-treated DBA/2N mice, and the *a** column depicts another species of P-450 formed after MC treatment of C57BL/6N mice.

Test compound	[Test compound]/[benzo[a]pyrene] ratio inhibiting enzyme activity 50%	
	*b**	*a**
1-(2-Isopropylphenyl)-imidazole	<0.01	1.0
1-(2-Cyanophenyl)-imidazole	<0.1	>10
SKF 525-A	<0.1	>10
Metyrapone	1.0	>10
17β-Estradiol	0.1	6
Δ⁹-tetrahydrocannabinol	0.4	4
DDT	1.0	>10
Pyridine	4	>10
n-Octylamine	2	>10
Cholecalciferol	5	>10
Aniline	10	>100
α-Naphthoflavone	>10	0.1
β-Naphthoflavone	>10	0.9
Lindane	>10	0.2
2,5-Diphenyloxazole	>10	0.4
Testosterone	>10	1.0
Isoproterenol	2	2
Norepinephrine	4	4
7,12-Dimethylbenz[a]-anthracene	1	1
Chlorpromazine	4	4

hibitors (Table 2). The first group of 14 compounds in Table 2 includes two phenylimidazole derivatives, metyrapone, and several amines, all of which incidentally display type II spectra upon combination with oxidized P-450. In this group are included all test compounds which inhibit the *b** hydroxylase activity at least 5 times as much as the *a** system. Differences in the absolute concentration at which a compound inhidits benzo[a]pyrene hydroxylation 50% are presumably related to the affinity of that compound for nonspecific as well as specific binding sites on the microsomal membranes. However, it is possible that the equilibrium binding constants and kinetic inhibition constants for these various compounds are not always related. Thus the effect of a compound on the rate of reduction of a form of P-450 may be different from its spectral effect. It has been reported that aliphatic primary amines and cyanide selectively bind low-spin P-450 (28), as is also true for metyrapone (17) and phenylimidazoles (29). Of interest within this first group, however, is that several type I substrates (e.g., SKF 525-A, 17β-estradiol, Δ⁹-tetrahydrocannabinol, diethylstilbestrol, and dexamethasone) compete preferentially with benzo[a]pyrene hydroxylation by the low-spin aryl hydrocarbon hydroxylase activity. Therefore we find no distinct correlation between a compound causing a type I or type II spectrum and its inhibitory effect on *b** hydroxylase activity. It would thus appear that compounds interacting with type *b** hydroxylase activity can exhibit either type I or type II difference spectra.

In the second group of Table 2, the five compounds which selectively inhibit *a** aryl hydrocarbon hydroxylase activity more than the *b** enzyme are also inducers of *a** activity. Both naphthoflavones are included among those aromatic hydrocarbons (6) which induce both hepatic and nonhepatic aryl hydrocarbon hydroxylase activity only in genetically responsive mice. Whereas 2,5-diphenyloxazole is apparently too toxic to induce the enzyme system *in vivo*,[3] this compound is a good inducer of the hydroxylase activity in secondary fetal hamster cultures, wherein most of the cells are nonhepatic (30). All these compounds produce type I spectra when mixed with oxidized P-450 *in vitro*.[3] We therefore suggest that α- and β-naphthoflavone, lindane, 2,5-diphenyloxazole, and testosterone share the common property of interacting with the newly formed high-spin P-450 species (i.e., type *a**) associated with aryl hydrocarbon hydroxylase induction by aromatic hydrocarbons.

Perhaps also belonging to this group are the chloro and bromo derivatives of 2-phenylbenzothiazole, which are also included in the class of aromatic hydrocarbons (6) able to induce hepatic and nonhepatic aryl hydrocarbon hydroxylase activity in *AhAh* or *Ahah* but not in *ahah* mice, and which also are type I substrates.[3] In the presence of 500 μM 2-(4′-chlorophenyl)benzothiazole or 2-(4′-bromophenyl)benzothiazole, the hydroxylase activity from PB-treated mice and especially the enzyme from MC-treated genetically responsive mice were lower in a pattern similar to that seen for the flavones, lindane, and 2,5-diphenyloxazole. These two halogenated derivatives, however, caused an apparent stimulation of benzo[*a*]pyrene hydroxylation in microsomes from control or MC-treated *ahah* mice. A possible explanation for a seemingly enhanced aryl hydrocarbon hydroxylase activity *in vitro* is that a lipophilic compound may bind to many nonspecific sites on the microsomal membrane, thereby making such sites less available for benzo[*a*]pyrene added subsequently. The later addition of the substrate then might result in more efficient metabolism of the polycyclic hydrocarbon. A second possible explanation for a seemingly enhanced hydroxylase activity *in vitro* is that the enzyme system is tightly coupled to an epoxide hydrase (31–33) so that some of the benzo[*a*]pyrene is converted to the *trans*-dihydrodiol (34, 35) rather than rearranged spontaneously to phenols (34). Hence inhibition of the epoxide hydrase but not aryl hydrocarbon hydroxylase *in vitro* may shunt more polycyclic hydrocarbon metabolites to the phenolic form, with which we equate the hydroxylase activity (6, 11). Preliminary studies[4] with these 2-phenylbenzothiazoles do not support the latter hypothesis.

Shown at the bottom of Table 2 are those compounds which inhibit both forms of aryl hydrocarbon hydroxylase activity equally. This group may also include 2-(4′-formylphenyl)benzothiazole and 2-*p*-toluenesulfonamidobenzothiazole, neither of which induce the hepatic or nonhepatic hydroxylase *in vivo* (6, 36). A fourth group of compounds includes those which did not appreciably affect either form of hydroxylase activity at concentrations 10-fold greater than the substrate: PB, allylisopropylacetamide, aminopyrine, cholesterol, *n*-hexane, cyclohexane, perfluoro-*n*-hexane, 1-butanol, and perhaps hexobarbital and laurate. Most of these chemicals interact with oxidized P-450 to produce type I difference spectra. PB and 1-butanol are reported (28) to bind preferentially to type *a* P-450. Perhaps these are two compounds that interact with the high-spin form of P-450, which distinctly differs from the *a** species highly specific for benzo[*a*]pyrene hydroxylation. We therefore raise the possibility that certain subsets of high- or low-spin P-450 exist. Last, we suggest that hexachlorobenzene represents a separate, fifth group in which aryl hydrocarbon hydroxylase activity from PB-treated animals is inhibited while the enzyme system from control or MC-treated mice is not at all affected.

The involvement of at least two forms of the same apparent monooxygenase activity has also been suggested by Staudinger and co-workers (37) and by Daly *et al.* (19) for acentanilide hydroxylation by liver microsomes, and by Pederson and Aust for aminopyrine *N*-demethylase (38). Recently three instead of two forms of aminopyrene *N*-demethylase activity have been described (39) with the use of differential inhibitory effects of the enzymes by dieldrin and DDT. Subclassifications of high- and low-spin P-450 appear likely to be identifiable. The other possibility is an intermediate spin state of 3/2 in addition to the high-spin ($S = 5/2$) and low-spin ($S = 1/2$) forms of oxidized P-450. Phthalocyanine complexes of ferric iron, for example, may exist in irregular planar or octahedral environments reported (40) likely to have three unpaired spins. To our knowledge, however, no biologically important iron has been detected with a spin state of 3/2. Since we now know the spin state with which increasing numbers of compounds interact, careful study of the preferential inhibition of other mixed-function oxygenases *in vitro* may elucidate the terminal P-450 sites involved in monooxygenation (41).

[4] F. Oesch, J. W. Daly, and D. W. Nebert, unpublished findings.

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