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On the Substrate Specificity of Cytochrome P450IIIA1

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SUMMARY

The instability of the solubilized/purified form, the lack of catalytic activity of the stabilized, macrolide-complexed form, and the compromised catalytic activity of the decomplexed form of steroid-inducible cytochrome P450IIIA1 motivated further investigations of the substrate specificity of this isozyme. A major complementary goal was to identify reactions utilizable as sensitive, specific diagnostic probes for the detection and partial characterization of this isozyme in tissues for which isolation and purification are not practical (e.g., extrahepatic, embryonic tissues, etc.). The approach utilized a combination of a specific, *purified* inducer, specific inhibitors including triacetyloleandomycin and inhibitory antibodies, and diagnostic probe substrates including the phenoxazone ethers, testosterone, warfarin, 2-acetylaminofluorene, estradiol-17β and benzo[*a*]pyrene. The results obtained indicated that steroid-inducible, rat hepatic P450IIIA1 would catalyze minimal or no *O*-dealkylation of methoxy-, ethoxy- or pentoxyphenoxazone but catalyzed rapid *O*-debenzylation of benzyloxyphenoxazone. Hydroxylation of testosterone was specific for the β face of the molecule at the 2-, 6-, 15- and 16-positions with no detectable conversion to androstenedione and minimal hydroxylation on the α face. Both the *R*- and *S*-enantiomers of warfarin were attacked at positions 9 and 10, and these reactions appeared to be specific to isozymes of the IIIA family. Aromatic hydroxylation of estradiol-17β was efficiently catalyzed, particularly at the 2-position. Hydroxylations of 2-acetylaminofluorene at positions 5 and 7 were catalyzed at relatively rapid rates, but *N*-hydroxylation of the same substrate was not catalyzed effectively. Hydroxylation of benzo[*a*]pyrene occurred preferentially at carbon 3 with much lesser activity at carbon 9 and little or no detectable attack at positions 7 or 1. The results indicated that the 2β- and 15β-hydroxylation of testosterone and the 10-hydroxylation of warfarin would serve as the most useful probes thus far available for detection of the presence of functional P450IIIA1 isozymes in tissues for which isolation and purification are impractical. The results also indicated a very broad, yet selective substrate specificity for the steroid-inducible P450IIIA1.

The cytochrome P450 superfamily represents a large number of hemoproteins whose principal function is the catalysis of the monooxygenation of drugs, steroids, lipids, and other small, liposoluble organic chemicals. The focus of the investigations in this report is on the substrate, position, and stereospecificity of a steroid-inducible, isozymic member of this superfamily, referred to by the recently standardized nomenclature (1) as P450IIIA1. This isozyme is distinguished from the closely homologous (90% with respect to amino acids) isozyme P450IIIA2 in that it is inducible by steroids and appears not to be constitutively present in male or female rat hepatic tissues at any age. P450IIIA2 is constitutively present in hepatic tissues of sexually mature male but not female rats. Members of this family of isozymes have commonly been referred to as P450p (2), P450PCN-E (3), P450PCN1 (4), P450PCNa (5), and P450PB2a (6). In this paper, reference will be made to P450IIIA1 as the steroid-inducible form. PCN and various synthetic glucocorticoids, of which DEX is the prototype, reportedly are capable of specifically inducing P450IIIA1 and/or closely related isozymes in rodent hepatic cells (1, 3–8).

Although several investigators have studied the substrate specificity of these isozymes, they have encountered a number of obstacles and problems not generally applicable to other isozymes of the P450 superfamily. Upon solubilization and partial purification, loss of catalytic activity occurs relatively rapidly (4–10). Various macrolide antibiotics, notably TAO, are converted by P450IIIA isozyme(s) to metabolites that bind to and stabilize the same isozyme(s) (11, 12), but the complexed form is not catalytically active per se. Displacement of the macrolide metabolite from the purified form with potassium ferricyanide or other oxidizing agents not only permits rapid degradation of the enzyme but also introduces complications in the interpretation of assays of activity by virtue of known and potential effects of the oxidizing agent and/or displaced macrolide metabolite on the reactions under study. Dialysis to remove these agents results in variable activity losses and it is conceivable that the entire procedure could alter substrate specificity. It seemed possible that a more satisfactory picture of the substrate specificity of this particular isozyme could be

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ABBREVIATIONS: PCN, pregnenolone-16α-carbonitrile; DEX, dexamethasone; TAO, triacetyloleandomycin; AAF, 2-acetylaminofluorene; BaP, benzo[*a*]pyrene ▱, 2-aminofluorene; PB, phenobarbital; HPLC, high-performance liquid chromatography; P450, cytochrome P450.

obtained with the freshly prepared, membrane-bound form as it exists within the confines of its natural lipophilic environment.

Thus, we have utilized an approach in which two separate sources of freshly prepared microsomes (hepatic microsomes from adult female Sprague-Dawley rats and from fetal rats of the same strain), each containing minimal or no detectable P450IIIA isozymes, were directly compared with the same two analogous preparations of microsomes in which relatively high concentrations of P450IIIA1 were present due to pretreatment of the rats with the *purified*, specific inducing agents, PCN or DEX. By utilizing highly purified inducers together with the use of inhibitory antibodies and TAO as specific inhibitors, we have provided important information regarding the regio- and/or stereoselectivity of steroid-inducible, P450IIIA1-catalyzed monooxygenation for a number of substrates that have attracted considerable attention as sensitive probes for specific P450 isozymes. These were AAF, estradiol-17β, a series of phenoxazone ethers, testosterone, and warfarin. When used together, this combination of approaches with specific probes provides a powerful tool for elucidating important aspects of the substrate specificity for this interesting isozyme family. The data also indicate that the most useful substrate probes for detection of P450IIIA1 in tissues containing minimal quantities of this (or closely related isozymes) are testosterone and R-warfarin. These, however, should be used in conjunction with specific inhibitors, of which TAO appears to be very useful.

Materials and Methods

Chemicals. Nonradiolabeled testosterone, estradiol-17β, unpurified DEX, NADPH, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, 3-methylcholanthrene, protein A-Sepharose CL-4B, and BaP were obtained from Sigma Chemical Co. (St. Louis, MO). AF and AF-9-one were synthesized in our laboratory according to methods described by Fletcher and Namkung (13) and Pan and Fletcher (14), respectively. AAF and 9-keto-AAF were subsequently synthesized by acetylation of AF and AF-9-one with acetic anhydride as earlier described (13). The purity of each of these compounds was >99% as assessed with analytical HPLC. All hydroxylated metabolites of AAF and BaP were acquired from the Chemical Repository of the National Cancer Institute. Isosafrole was purchased from ICN Pharmaceuticals (Plainview, NY); phenobarbital (PB) was obtained as the pure powdered sodium salt from the University Hospital Pharmacy (Seattle, WA); [9-^{14}C]AAF (52 mCi/mmol, 98% purity) and [7,10-^{14}C]BaP (47.2 mCi/mmol, 97% purity) were purchased from Amersham Corp. (Arlington Heights, IL). [9-^{14}C]AAF was further purified (>99.5%) by HPLC using a Whatman Partisil ODS-2 Magnum (M9 10/25) column and eluting isocratically with methanol/water (80:20), and [7,10-^{14}C]BaP was purified (>99%) with preparative, reverse-phase HPLC on a Whatman Partisil ODS-2 Magnum (M9 10/25) column with a linear methanol/water gradient (80–100%, 30 min). PCN was obtained from three separate sources: as a generous gift from Searle Laboratories (Chicago, IL); as a purchase from The Upjohn Company (Kalamazoo, MI); and from chemical synthesis in our own laboratory. PCN was synthesized and purified (melting point, 231–232°) according to methods described by Mazur and Cella (15). Analyses with analytical HPLC indicated that the Upjohn preparation contained a relatively large amount (>7%) of several impurities and that the Searle preparation contained slightly lesser (5–7%) quantities of contaminants. The PCN synthesized in our laboratory was purified such that the final preparation was >99.5% pure by analytic HPLC. Similarly, HPLC analyses indicated that the DEX supplied by Sigma was only 87% pure (m.p. 62–65°). We recrystallized the material from 95% ethanol (m.p. 66–68°) and subsequent HPLC analysis indicated a purity of 99.5%.

Hydroxylated testosterone metabolites were kindly supplied by Professor D. N. Kirk from the MRC Steroid Reference Collection (London, England), except that 15β-hydroxytestosterone was supplied as a generous gift from Searle Research Division (Skokie, IL). [2-^{3}H]- and [4-^{3}H]estradiol-17β were prepared in our laboratory as described earlier (16). TAO was kindly provided by the Roerig Division of Pfizer Pharmaceuticals (New York, NY). The methoxy, ethoxy, pentoxy, and benzyloxy ethers of phenoxazone were synthesized and purified in our laboratory in accordance with methods described by Mayer *et al.* (17) and Klotz *et al.* (18), respectively. Melting points were in good agreement with the published literature values (18). Resorufin and racemic warfarin were obtained from Aldrich Chemical Co. (Milwaukee, WI) and phenoxazone was supplied by Dr. A. E. Rettie, Department of Medicinal Chemistry, University of Washington. The *R*- and *S*-warfarin enantiomers and various warfarin metabolites were kindly provided by Dr. William Trager of the same department. [4-^{14}C]Testosterone (59.6 mCi/mmol, 99% pure) and [9-^{14}C]warfarin (racemic; 46 mCi/mmol, 99% pure) were each obtained from Amersham. Purities of these labeled compounds were checked and verified with analytical HPLC. All other chemicals utilized were of the highest quality available commercially.

Enzyme sources. Sexually mature female Sprague-Dawley (Wistar-derived) rats (220–260 g) were obtained from Tyler Laboratories (Bellevue, WA) and were housed in the University Vivarium with food (Purina Rat Chow) and water *ad libitum*. Two to three animals were placed in single cages which contained crushed corncob bedding material (Bed-'o-cobs, Sanicel) and were kept on a 14-hr light, 10-hr dark lighting schedule. Primigravida pregnant as well as nonpregnant animals were treated with PCN or DEX as inducing agents according to the following schedules. Inducers were suspended in corn oil and administered intraperitoneally (PCN, 80 mg/kg; DEX, 100mg/kg) once daily for 4 days. Pregnant rats were given the same doses but as divided, twice daily injections initiated on the morning of day 16 of pregnancy (the morning after copulation is designated as day 0). Fetuses were removed 24 hr after the last injection, and fetal livers from three to four dams were pooled and homogenized in a Potter homogenizer with a Teflon pestle. Homogenates were centrifuged at 9,000 × *g* for 10 min, and the resultant supernatant was centrifuged at 104,000 × *g* for 1 hr to sediment the microsomal fraction. Livers of nonpregnant females were homogenized and centrifuged at 9,000 × *g* for 20 min with subsequent sedimentation of the microsomal fraction at 104,000 × *g* for 1 hr. Sedimented microsomes were washed, resedimented, suspended in buffer, and utilized immediately as enzyme sources except where otherwise indicated.

Purified P450s and P450 antibodies. Cytochrome P450IIIA was purified from male rat liver microsomes in accordance with methods described by Elshourbagy and Guzelian (9) and also as the TAO-complexed form (probably a mixture of IIIA1 and IIIA2 and hereafter referred to as P450IIIA1/2) by the method described by Wrighton *et al.* (19). In order to exclude antigenic proteins that may have been present in minute quantities, the TAO-complexed preparation was further purified by loading 0.125-mg quantities onto a preparative, 10–20% gradient, sodium dodecyl sulfate gel. The only evident band (after Coomassie Blue staining) was excised and homogenized in 0.5 ml of potassium phosphate-buffered normal saline (pH 7.4). The homogenized sample was sonicated with 0.5 ml of Freund's complete adjuvant and used as a source of antigen for generation of P450IIIA antibodies. Antibodies were generated by injecting the complexed form subcutaneously into female New Zealand White rabbits. Injections (in Freund's incomplete adjuvant) were repeated after 1 and 2 months, and blood was withdrawn 2 weeks after the final injection. An IgG-enriched fraction was prepared (20) by passage of sera through a protein-A-Sepharose CL-4B column.

P450s IA1, IA2, and IIB1/2 were also prepared from rat liver microsomes exactly according to methods described by Ryan *et al.* (21, 22), except that the final immunopurification step for P450IA2 was not carried out, and no antibodies were elicited for this isozyme. Other

TABLE 1

Rates of dealkylation and debenzylation of phenoxazone ethers in freshly prepared hepatic microsomes of adult female rats (experiments 1–6) or rat fetuses (experiments 7 and 8) (treated with steroidal inducing agents

Results are expressed as mean ± SD (N = 5–7). Conversion of the various phenoxazone ether substrates to resorufin was assayed fluorimetrically as described under "Materials and Methods." PCN and DEX were obtained commercially or synthesized and purified as described under Materials and Methods. ND indicates that activities were below the level of detectability (<0.05 pmol/min). Reaction cuvettes contained 0.02–0.2 mg of microsomal protein, 10 nmol of substrate dissolved in 10 μl of dimethyl sulfoxide, 1.0 μmol of NADPH, and potassium phosphate buffer (0.1 M, pH 7.4) to bring the total volume to 1.0 ml. Reactions were run at 37°.

Inducing Agent	Specific Activity			
	Demethylation	Deethylation	Depentylation	Debenzylation
	pmol/mg protein/min			
1. None	16.3 ± 3.7	48.5 ± 4.2	1.2 ± 0.3	10.1 ± 2.3
2. PCN, Upjohn	8.9 ± 1.9	120.5 ± 7.8	3.5 ± 1.0	64.2 ± 5.9
3. PCN, Searle	7.4 ± 2.1	109.1 ± 9.4	2.8 ± 0.7	70.3 ± 8.0
4. PCN, purified	4.9 ± 1.3	46.1 ± 5.4	1.3 ± 0.4	68.2 ± 5.2
5. DEX, Sigma	9.9 ± 2.6	104.9 ± 10.8	25.9 ± 4.6	134.2 ± 9.5
6. DEX, purified	4.2 ± 0.8	107.0 ± 9.2	32.5 ± 5.1	207.0 ± 15.9
7. None (fetal)	ND	ND	ND	0.3 ± 0.2
8. PCN, purified (fetal)	ND	ND	ND	2.6 ± 0.5

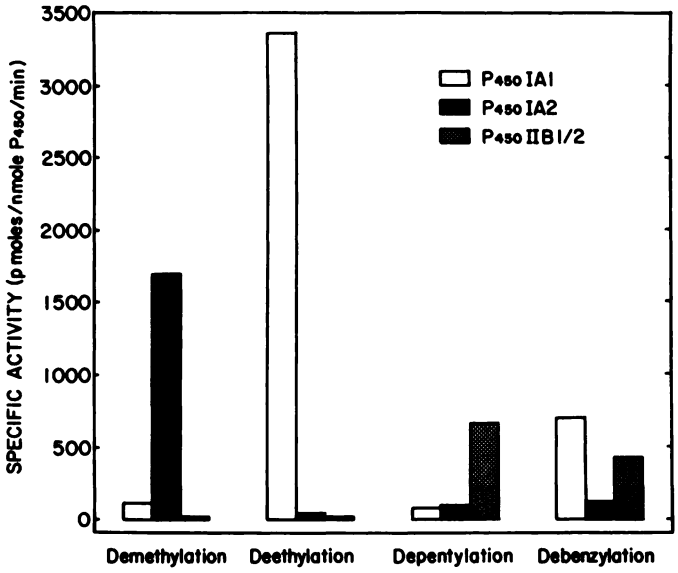

Fig. 1. Dealkylase and debenzylase activities of purified P450s in reconstituted systems. Reaction vessels contained 0.02–0.1 nmol of P450, 0.01–0.02 nmol of NADPH-cytochrome P450 reductase, 10 nmol of substrate, 1 μmol of NADPH, and 40 nmol of dilauroylphosphatidylcholine in 0.1 M potassium phosphate buffer, pH 7.4.

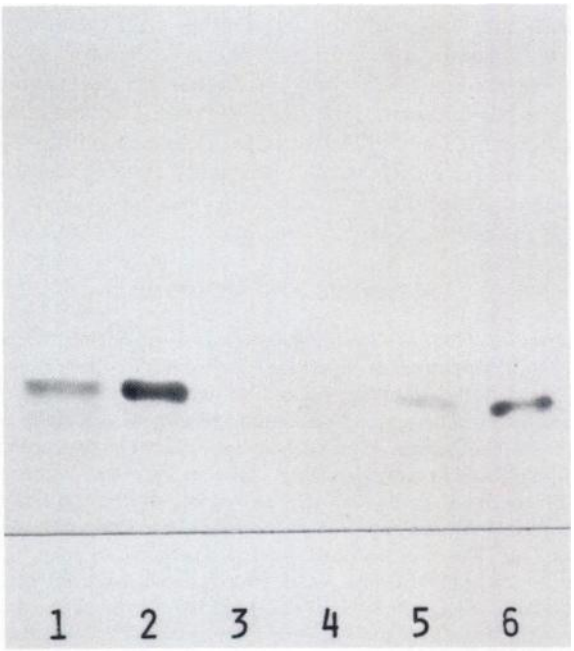

Fig. 2. Immunoblot of purified P450IIB1/2 and hepatic microsomes from adult female rats. Shown are microsomal protein from rats induced with purified DEX (20 μg, *lane 1*), rats induced with PB (7.5 μg, *lane $*), untreated rats (20 μg, *lane 3*), rats induced with purified PCN (20 μg, *lane 4*), rats induced with unpurified PCN from Upjohn (20 μg, *lane 5*), and 0.1 μg of purified P450IIB1/2 (*lane 6*).

commonly utilized names for P450IA1 are $P450_c$ and BNF-B; for P450IA2 are $P450_d$ and ISF-G; for P450IIB1 are $P450_b$, PBB and PB-4; for P450IIB2 are $P450_e$, PBD, and PB-5. In routine separations, the DE-52 column did not provide complete separation of IIB1 from IIB2, but no further attempt was made to separate IIB1 from IIB2 since they are 97–98% homologous and the preparation is referred to as P450IIB1/2. Catalytic activities of IIB1 are normally much higher than those of IIB2 but, qualitatively, quite similar. The purified isozymes had specific contents of 13–17 nmol of P450/mg of protein. Electrophoresis yielded single protein staining bands on sodium dodecyl sulfate-polyacrylamide gels. NADPH-cytochrome P450 reductase was purified from hepatic microsomes of PB-induced rats by methods described by Shepard *et al.* (23). The reductase preparation exhibited a specific activity of 57.2 μmol/mg/min at 24° in a solution containing 0.33 M potassium phosphate buffer (pH 7.4), 10 mM EDTA, 3 mM $MgCl_2$, 0.1 mM KCN, 0.05 mM cytochrome *c*, and 0.1 mM NADPH. Antibodies were raised against P450s IA1 and IIB1/2 by the method described by Thomas *et al.* (24). Polyclonal antibodies known to exhibit considerable immunologic cross-reactivity include IA1 with IA2, IIB1 with IIB2, and IIIA1 with IIIA2. However, cross-reactivity *among* the purified members of these three groups is not normally encountered. Analyses performed with enzyme-linked immunosorbent assays (25) and with Western blots (26) indicated no detectable cross-reactivity among antibodies raised against IA1, IIB1/2, and IIIA1/2. Antibodies were not raised against IA2. (For Western blots, 5% nonfat dry milk was used to block nonspecific binding, and the substrate used for color development was 4-chloro-1-naphthol.) For utilization as potential enzyme inhibitors, IgGs were preincubated with the enzyme sources at 25° for 15 min with a ratio of 0.5–10 mg of IgG/nmol of P450 as determined in preliminary experiments with a range of concentrations. IgG titers were determined with the enzyme-linked immunosorbent assay. When tested against P450s IIIA1/2, IIB1/2, IA1, or P450-oxidoreductase, absorbance associated with P450IIIA1/2 remained significantly greater than that for the latter three at a final concentration of 400 ng/ml. This concentration corresponds to an approximate serum dilution of 1:10,000.

TABLE 2

Effects of induction with PCN on conversion of warfarin to various metabolites by hepatic microsomal preparations from adult female and fetal rats

Results are expressed as mean ± SD (N = 3–5). Quantities of metabolites generated were assessed by hplc analyses as described under Materials and Methods. Linearity of conversion as a function of time and protein concentration was verified in preliminary experiments. Retention time of R-warfarin was 20.1 min. ND indicates that quantities generated were below the limits of detectability (150 pmol) (UV); 24 pmol (radiometric) of metabolite/reaction vessel). Radioactive racemic warfarin was utilized in experiments with fetal tissues. Reaction vessels contained 0.4 μmol of R-warfarin (adults) of 2.5 μCi of racemic warfarin (fetuses), 1–2 mg of microsomal protein, 1.0 μmol of NADPH, 2.0 μmol of glucose-6-phosphate dehydrogenase and sufficient potassium phosphate buffer (0.1 M, pH 7.4) to bring the total volume to 1.0 ml. Incubations were carried out at 37° for 15 min (adult) or 120 min (fetuses) under 100% O_2.

R-Warfarin Metabolite	Retention Time	Enzyme Source			
		Adult Females (Control)	Adult Females (PCN-Induced)	Fetuses (Controls)	Fetuses (PCN-Induced)
	min	*pmol/mg protein/min*			
Dehydro	3.5	28 ± 5	342 ± 57	ND[a]	ND[a]
4′-Hydroxy	7.8	31 ± 8	47 ± 16	ND	ND
6-Hydroxy	9.6	43 ± 3	59 ± 6	ND	ND
10-Hydroxy	10.8	ND	24 ± 3	ND	0.73 ± 0.18
8-Hydroxy	12.6	67 ± 6	85 ± 15	ND	ND
7-Hydroxy	14.1	352 ± 29	553 ± 49	ND	0.51 ± 0.13

[a] Relatively large amounts of radioactivity eluting with the solvent front prevented accurate quantitation of the dehydro metabolite with fetal tissues.

Enzyme assays. Enzymic conversion of testosterone to androstenedione and various hydroxylated metabolites was assessed with HPLC in accordance with slight modifications of the procedures described by Wood *et al.* (27) except that a Rainin Microsorb C_{18} (5 μ, 150 × 4.6 mm) column was employed in this study. Retention times of metabolites are provided in Table 3. For analyses with adult female liver, UV detection of eluted metabolites was utilized. For experiments with fetal livers, radiolabeled [^{14}C]testosterone was utilized as substrate, 18-sec fractions were collected from the HPLC column, and metabolites were quantitated via liquid scintillation counting. Hydroxylation of the aromatic ring of estradiol-17β was assayed by methods described earlier (28). Conversion of R- and S-warfarin by adult liver to oxidized metabolites followed essentially the same procedures as those described by Kaminsky *et al.* (29). Bioconversion of radiolabeled racemic warfarin to metabolic products by fetal livers utilized the same procedure except that eluted fractions were collected and radiolabeled metabolites quantitated via scintillation spectrometry. Measurements of the conversion of AAF and BaP to oxidized metabolites were also performed with HPLC analyses by the methods described earlier by Faustman-Watts *et al.* (30) and by Dean *et al.* (31), respectively. Conversion of the four phenoxazone ethers to resorufin was followed with the continuous fluorimetric assays as earlier described by Rettie *et al.* (32). For each of these assays, we verified in preliminary experiments that the reactions were zero order with respect to substrate and were linear with respect to time and protein concentrations. Incubation conditions for individual assays are described in tables and figures. Proteins were measured with the methods described by Bradford (33) using crystalline bovine serum albumin as a standard.

Results and Discussion

Analyses of the effects of steroid induction on rates of dealkylation and debenzylation of phenoxazone ethers (Table 1) showed that the purity of the inducing agent significantly influenced the results obtained. With unpurified PCN, 2–3-fold increases in deethylation were observed whereas this activity was not increased when the purified steroid was utilized. This result suggested that some of the impurities would induce P450IA1 or related isozymes since this P450 is highly efficient for catalysis of the deethylation reaction (4, 34). Purified PCN did not elicit a significant increase in dependentylase activity whereas use of either of the unpurified PCN preparations results in 2–3-fold increases in this activity. These results suggested that the unpurified preparations also contained varying quantities of "phenobarbital-type" inducing agents, since phenobarbital induces isozymes (e.g., IIB1) known to efficiently catalyze the dependentylation reaction (4, 35). However, the purified and unpurified PCN preparations each produced marked increases (9–10-fold) in debenzylation, suggesting strongly that benzyloxyphenoxazone is a reasonably good substrate for P450IIIA1. In addition, treatment with purified PCN resulted in marked decreases (70%) in demethylation. This contrasted with lesser (45–55%) decreases elicited by the unpurified preparations. The decreases in demethylation activity may have resulted from repression of P450IA2 since the reconstituted isozyme preferentially catalyzed the demethylation reaction (Fig. 1). In reconstituted systems with P450IA1, IA2, and IIB1/2, it was found that each of these preparations catalyzed relatively rapid debenzylation reactions. Also, microsomes from

TABLE 3

Effects of induction of P-450IIIA1 with PCN on conversion of testosterone to androstenedione and hydroxylated metabolites by hepatic microsomal preparations from adult females and fetuses near term

Results are expressed as mean ± SD (N = 3). Metabolites generated were quantitated by HPLC analyses as described and Materials and Methods. Linearity of conversion as a function of time and protein concentration was verified in preliminary experiments. Retention time of testosterone was 22.3 min. ND indicates that quantities of metabolites generated were below the limits of detectability (24 pmol of metabolite/reaction vessel, fetuses; 150 pmol, adults) for the respective assays. Reaction vessels contained 1.0 pmol of NADPH, 3 μmol of $MgCl_2$, 50 μmol of sucrose, 250 nmol (adults) or 1 μCi (fetuses) of testosterone in 20 μl of ethanol, 1–4 mg of microsomal protein, and potassium phosphate buffer (0.1 M, pH 7.4) to bring the total volume of 1.0 ml. Incubations were carried out at 37° for 15 min (adults) or 120 min (fetuses) under 100% O_2.

Testosterone Metabolite	Retention Time	Adult Female (control)	Adult Female (PCN-induced)	Fetus (control)	Fetus (PCN-induced)
	min		*pmol/mg protein/min*		
6α-OH	5.5	20 ± 7	15 ± 5	0.08 ± 0.04	0.23 ± 0.09
15β-OH	6.2	ND	1108 ± 221	2.45 ± 0.61	15.37 ± 2.18
7α-OH	7.5	348 ± 46	418 ± 63	0.05 ± 0.03	1.59 ± 0.71
6β-OH	8.2	172 ± 34	2822 ± 312	4.18 ± 1.24	739.8 ± 9.32
16α-OH	9.9	103 ± 24	488 ± 91	6.96 ± 2.51	12.17 ± 3.03
Unidentified	12.1	ND	ND	0.58 ± 0.11	0.51 ± 0.31
16β-OH	13.1	ND	244 ± 37	1.33 ± 0.40	7.12 ± 1.86
2α-OH	14.5	13 ± 7	28 ± 13	0.20 ± 0.07	1.14 ± 0.36
2β-OH	15.1	ND	906 ± 82	1.17 ± 0.60	31.65 ± 5.21
Androstenedione	20.6	ND	ND	1.08 ± 0.49	0.62 ± 0.13

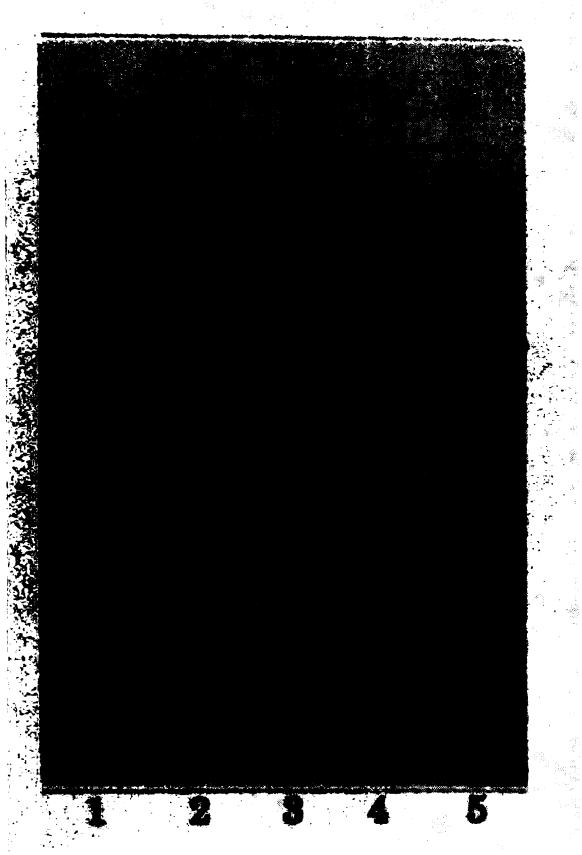

Fig. 3. Immunoblot of purified P450IIIA1/2 and hepatic microsomes from adult female and fetal rats. Shown are 20 μg of microsomal protein from fetal rats induced with purified PCN (*lane 1*), 20 μg of microsomal protein from untreated fetal rats (*lane 2*), 1.0 μg of purified P450IIIA1/2 (*lane 3*), 20 μg of microsomal protein from adult female rats induced with purified PCN (*lane 4*), and 20 μg of microsomal protein from untreated adult females. (*lane 5*) Fetal rats were sacrificed on day 20 of gestation.

TABLE 4

Release of tritium from tritium-labeled estradiol-17β in hepatic microsomes of adult female and fetal rats after treatment with PCN

Estimated relative rates of 2- and 4-hydroxylation were obtained by measuring tritium displacement from [2-^{3}H]estradiol-17β and [4-^{3}H]estradiol, respectively, as described under Materials and Methods. Results are given as mean ± SD (n = 3–5). Linearity of the reactions with respect to time and protein concentrations was verified in preliminary experiments. Limits of detectability were 41 pmol/reaction vessel. Incubation vessels contained 500 nmol of substrate in 20 μl of ethanol, 0.2–2.0 mg of microsomal protein, 8.8 μmol of glucose 6-phosphate, 2 units of glucose-6-phosphate dehydrogenase, 0.35 μmol of NADPH, and potassium phosphate buffer (0.1 M, pH 7.4) to bring the total volume to 1.0 ml. Incubations were carried at 37° for 15 min (adults) or 120 min (fetuses) under 100% O_2.

Hepatic Enzyme Source	Specific Activity	
	[2-^{3}H]Estradiol-17β	[4-^{3}H]Estradiol-17β
	pmol/mg protein/min	
Adult female (control)	213 ± 32	116 ± 25
Adult female (PCN-induced)	2470 ± 211	1312 ± 201
Fetus (control)	2.3 ± 0.8	0.5 ± 0.2
Fetus (PCN-induced)	78.2 ± 6.2	11.4 ± 2.7

untreated adult female rats, which lack many inducible P450 isozymes (e.g., IA1, IIB1, and IIIA1) as well as various male-specific isozymes (e.g., $P450_h$, $P450_g$, and P450IIIA2), catalyzed relatively rapid debenzylation reactions. These observations suggest that many if not most P450 isozymes will catalyze debenzylation of benzyloxyphenoxazone. Thus, even though benzyloxyphenoxazone is a relatively good substrate for P450IIIA1, its usefulness as a probe substrate for this isozyme is limited primarily to employment as an initial screen probe. The ease and sensitivity of the debenzylation assay, however, suggest that it may be a highly useful probe for detection of xenobiotic-biotransforming P450s in general in extrahepatic or embryonic tissues. Support for this idea was also given in an earlier publication (32).

Experiments with DEX as inducer (Table 1) indicated that even the purified glucocorticoid produced marked increases in the dependentylation of pentoxyphenoxazone. This strongly suggested that purified DEX induces a broader spectrum of P450 isozymes than purified PCN. An attempt also was made to determine whether P450IIB1/2 could be detected immunologically after DEX induction (Fig. 2). The data corroborated the results with pentoxyresorufin and indicated that purified DEX is less specific than purified PCN as an inducer of P450IIIA1 in rats. The data of Simmons *et al.* (7) also suggested differences, but they measured a 40% decrease in immunoquantifiable P450IIB1, an isozyme that catalyzes dependentylation very efficiently. Yamazoe *et al.* (36), however, observed marked increases in both immunoquantifiable IIB1 and pentoxyphenoxazone dependentylase as well as testosterone 16α-hydroxylase activities. Reasons for these differences are presently unexplained but may reflect differences in the composition of commercially obtained, unpurified DEX preparations and reemphasize the need for utilization of purified inducers. For all subsequent experiments we utilized purified PCN as inducer except where otherwise noted.

In experiments with *R*-warfarin as substrate (Table 2), induction with purified PCN resulted in marked increases in generation of the dehydro and 10-hydroxylated metabolites but only minimal increases in hydroxylation at the 4′-, 6-, 7-, and 8-positions. Experiments with *S*-warfarin yielded results qualitatively very similar to those obtained with the *R*-isomer, with marked PCN-elicited increases in the generation of the dehydro and 10-hydroxy metabolites and minimal or no changes in quantities of metabolites hydroxylated at the other four positions. The *R*-isomer was attacked somewhat more efficiently than the *S*-isomer with *R*/*S* ratios for the dehydro and 10-hydroxy metabolites of 1.7 and 1.9, respectively.

Reaction systems utilizing untreated, control fetal livers as enzyme source and racemic radiolabeled warfarin as substrate (see "Materials and Methods") did not generate detectable quantities of metabolites hydroxylated at any of these positions. Transplacental induction with purified PCN resulted in low but readily measurable hydroxylation at carbons 10 and 7. The dehydro metabolite could not be quantitated in fetal preparations because relatively high and variable quantities of radioactivity eluting with the solvent front overlapped the elution position of the dehydro metabolite. Western blots (Fig. 3) also showed that P450IIIA was inducible in the fetal livers.

With regard to substrate specificity, the results reported here tended to agree with results reported by Kaminsky and collaborators (4, 5, 29) in which selective attacks at warfarin carbons 9 (which spontaneously converts to the dehydro) and 10 by P450IIIA were observed. We performed additional experiments in an attempt to further confirm the 10-hydroxylation reaction. We collected HPLC-eluted samples (see "Materials and Methods") of the putative 10-hydroxy metabolite, concentrated, and subjected them to analysis with gas chromatography-mass spec-

TABLE 5

Effects of induction of P450IIIa1 with purified PCN on conversion of AAF to oxidized products by hepatitis microsomal preparations from adult females and fetuses near term

Results are expressed as mean ± SD (N = 3–5). Conversion to AAF to the indicated metabolites was determined by HPLC analyses as described under Materials and Methods. Linearity of conversion as a function of time and protein concentration was verified in preliminary experiments. Retention time of AAF was 23.8 min. ND indicates that quantities of metabolites generated were below the limits of detectability (30 pmol/reaction vessel). Reaction flasks contained 1.0 μmol of NADPH, 2.0 μmol of glucose 6-phosphate, 2 units of glucose-6-phosphate dehydrogenase, 0.5–3.0 mg of microsomal protein, 40 nmol of AAF (0.5 μCi) in ethanol, and potassium phosphate buffer to a final volume of 1.0 ml. Incubations were carried at 37° for 15 min (adults) and 120 min (fetuses) under 100% O_2.

AAF Metabolite	Retention Time	Adult Female (Control)	Adult Female (PCN-Induced)	Fetus (Control)	Fetus (PCN-Induced)
	min	*pmol/mg protein/min*			
Unidentified 1	2.5	3.1 ± 2.3	11.2 ± 3.9	0.2 ± 0.1	ND
Unidentified 2	3.5	2.0 ± 1.1	5.9 ± 2.2	0.1 ± 0.0	1.5 ± 0.9
7-Hydroxy	5.2	30.8 ± 5.4	283.6 ± 43.1	0.2 ± 0.1	16.2 ± 3.1
9-Hydroxy	6.7	2.6 ± 1.3	26.5 ± 7.3	0.3 ± 0.1	14.8 ± 4.7
5-Hydroxy	7.8	4.1 ± 1.2	44.7 ± 8.2	0.4 ± 0.2	6.1 ± 1.9
9-Keto	12.7	0.5 ± 0.3	5.4 ± 2.6	0.2 ± 0.1	0.2 ± 0.2
3-Hydroxy	16.0	1.1 ± 0.6	1.5 ± 0.5	0.4 ± 0.2	0.7 ± 0.3
1-Hydroxy	18.1	0.8 ± 0.3	6.0 ± 2.7	ND	1.6 ± 0.8
N-Hydroxy	20.6	0.2 ± 0.1	ND	ND	ND
Unidentified 3	31.4	4.2 ± 3.4	21.1 ± 4.0	1.0 ± 0.5	2.5 ± 1.3

trometry by the methods described by Rettie *et al.* (32). The results obtained supported the identity of the eluted peak as the 10-hydroxywarfarin metabolite.

Induction of adult female rats with purified PCN resulted in profound increases in quantities of testosterone metabolites coeluting with standards hydroxylated at the 2β-, 6β-, 15β-, and 16-β-positions (Table 3). Hydroxylation reactions occurring on the α face of the steroid molecule (2α, 6α, 7α, and 16α) exhibited much lesser increases (or even slight decreases) after PCN induction than in the corresponding control preparations and the differences were not statistically significant by Student's t test ($p > 0.05$) except in the case of 16α-hydroxylation where a 4–5-fold increase was observed. The marked increase in 15β-hydroxylation supports the results of several previous studies. Other results with testosterone are also consistent with data reported by Waxman *et al.* (6), Gonzalez *et al.* (4), and Graves *et al.* (5) although we did observe a statistically significant ($p < 0.05$) increase in 16α-hydroxylation, not reported by other investigators. We also did not observe an increase in 18-hydroxylation as did Sonderfan *et al.* (37), but this minor metabolite was not investigated in our study.

The results obtained with fetal hepatic tissues (Table 3) were somewhat unexpected and suggested that occurrence and regulation of steroid-hydroxylating enzymes in the fetal rat liver may not be predictable from observations on mature rats. Hydroxylations of testosterone at the 15β- and 16β-positions occurred to a greater extent in control fetal livers than in control adult female livers. The magnitude of induction by PCN was substantially less in the fetal liver than in the adult. The 7α-hydroxylation reaction was only minimally affected in the adult but increased by more than 30-fold in the fetus, suggesting the possibility that PCN may induce P450IIA1 in fetal livers. (This isozyme is also commonly referred to as $P450_e$, UTF, and 3). Hydroxylation at the 2α-carbon was also increased 5–6-fold in the fetal liver, suggesting the possibility that PCN may be capable of transplacentally inducing $P450_h$ (a male-specific isozyme not yet named systematically and present in hepatic microsomes of adult male rats) or homologous isozyme(s) in fetal rats. PCN also elicited marked increases in rates of hydroxylation at the 6β-, 16β-, 15β-, and 2β-positions, in consonance with the results obtained with female adults and with the concept that P450IIIA1 is induced by PCN in fetal livers. Overall, the results with testosterone as substrate strongly suggest a different complement of constitutive P450 isozymes in fetal versus adult female hepatic tissues and that, in the fetal liver, PCN is capable of transplacentally inducing P450(s) that catalyze hydroxylation of testosterone on the α face at carbons 2 and 7 and, to a lesser extent, at carbons 6 and 16. A considerable amount of additional research will be required to clarify these issues. Taken together, however, the results obtained support the view that P450IIIA1 attacks testosterone primarily on the β face of the molecule and preferentially at carbons 2, 6, 15, and 16. The immunologic data presented in Fig. 3 also support this concept.

Studies with estradiol-17β as substrate indicated that P450IIIA1 catalyzes efficient attack on the aromatic ring of the

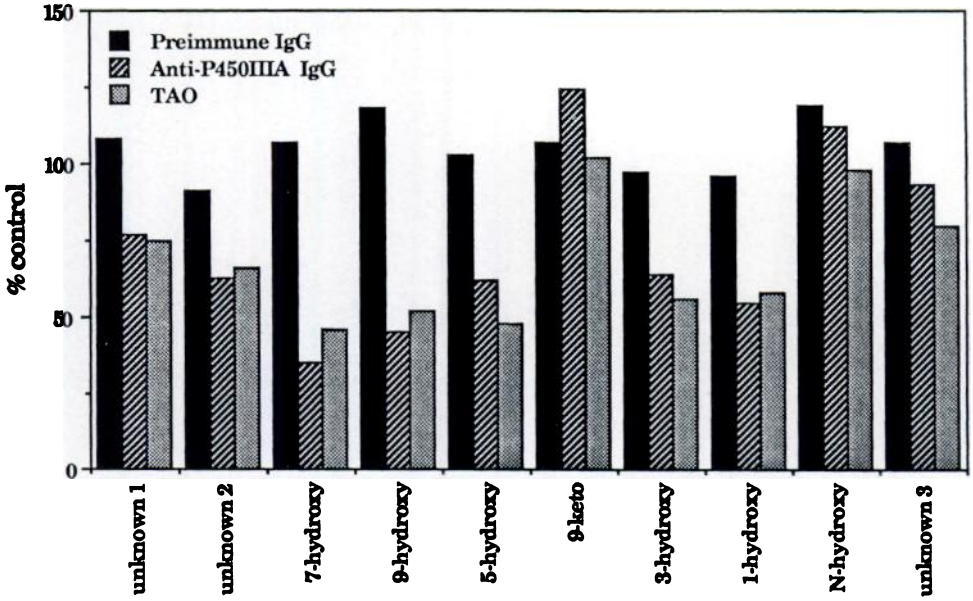

Fig. 4. Effects of preimmune IgG, anti-P450IIIA1/2 IgG, and TAO on conversion of AAF to various hydroxylated metabolites. Hepatic microsomes from adult, female Sprague-Dawley rats, pretreated with purified PCN as inducer (see Materials and Methods) were utilized as enzyme source. In analogous experiments performed with microsomes from noninduced female rats, none of the three substances exhibited significant inhibitory effects. For incubation conditions and inhibitor concentrations, see Table 5–7. Results are given as percentages of control (no addition) for each metabolite peak.

TABLE 6

Metabolites of BaP generated by freshly prepared hepatic microsomes from adult female rats and the effects of induction with purified preparations of PCN or DEX

Results are expressed as mean ± SD (N = 3–4). A combination of various HPLC separation systems (see Materials and Methods) was utilized to separate and quantitate the listed metabolites. Thus, retention times are not listed. Generation of the 4,5-oxide, 1-hydroxy, and 6,12-dione in these experiments was below the limits of detectability (60 pmol/flask) under the reaction conditions used. Incubation vessels contained 1.0 μmol of NADPH, 2.0 μmol of glucose 6-phosphate, 2 units of glucose-6-phosphate dehydrogenase, 0.1–0.5 mg of microsomal protein, 80 nmol of substrate (0.5 μCi) in 20 μl of acetone and potassium phoshpate buffer (0.1 M, pH 7.4) to a total volume of 1.0 ml. Incubations were carried at 37° for 15 min under 100% O_2.

Metabolite	Enzyme Source		
	Adult Female (Control)	Adult Female (PCN-Induced)	Adult Female (DEX-Induced)
	pmol/mg protein/min		
9,10-Diol	0.40 ± 0.11	0.09 ± 0.05	0.15 ± 0.07
4,5-Diol	0.25 ± 0.06	0.64 ± 0.23	0.51 ± 0.13
7,8-Diol	0.60 ± 0.17	0.43 ± 0.23	0.30 ± 0.08
4,5-Dione	0.27 ± 0.10	0.41 ± 0.12	0.16 ± 0.03
1,6-Dione	0.12 ± 0.04	0.56 ± 0.16	0.55 ± 0.11
3,6-Dione	0.35 ± 0.10	1.74 ± 0.36	1.59 ± 0.40
9-Hydroxy	0.31 ± 0.08	0.67 ± 0.19	0.55 ± 0.15
7-Hydroxy	0.22 ± 0.06	0.19 ± 0.05	0.25 ± 0.07
3-Hydroxy	1.06 ± 0.32	3.97 ± 0.58	3.61 ± 0.49

steroid molecule (Table 4). The method utilized does not provide absolute position specificity because hydroxylation at carbon 2 can result in some release (via tautomerization or other reactions) of tritium from carbon 4 and vice versa (28, 38). Jellinck *et al.* (39, 40) have recently provided evidence to indicate that tritium is lost from carbon 2 primarily during 2-hydroxylation and from carbon 4 predominantly after catecholestrogen formation and during binding to GSH, proteins, or other nucleophiles. With those considerations, the data presented in Table 4 indicate that P450IIIA1 catalyzes aromatic hydroxylation of estradiol at carbon 2 in preference to carbon 4 and that this steroid molecule is a reasonably good substrate for the IIIA1 isozyme. The results are in substantial agreement with those of Dannan *et al.* (41) and earlier preliminary results from this laboratory (28). However, the earlier data also demonstrated that isosafrole-induced (IA2) and male-specific (h, also known as UTA, 2_C, and RLM-5) isozymes also catalyzed efficient 2-hydroxylation of estradiol, thus indicating that this substrate is not a specific P450IIIA probe. Again, because of the extremely high sensitivity and ease of assay, it may be very useful as a preliminary probe.

Results obtained with AAF as substrate (Table 5) contrasted with those reported by previous investigators (10) who utilized partially purified, reconstituted P450IIIA1 preparations in attempts to investigate this substrate. The discrepancy appears likely to be due to the catalytic inactivity of the partially purified cytochrome. It was of interest in our experiments that the freshly prepared, PCN-induced preparations exhibited profoundly greater activities than the corresponding control preparations for all assayed positions on the AAF molecule except at the nitrogen (N-hydroxylation) and at carbon 3. Lesser increases were also observed at carbon 1 and the greatest increases occurred at carbons 7, 5, and 9 in that order. Increases were also observed for three unidentified metabolites, but the magnitude of these increases was generally lower than for those observed at carbons 7, 5, and 9. With AAF as substrate, the results obtained with fetal tissues (Table 5) were in good agreement with those observed in adult females and supported the view that P450IIIA1 is capable of attacking AAF at several positions on the molecule but with weaker attack on or near the amide nitrogen. Inhibition with TAO and anti-P450IIIA1/2 antibodies (Fig. 4) also supported these ideas. It is known that various other P450 isozymes are also efficient catalysts of the hydroxylation of AAF (10). P450IA1 was highly efficient for ring hydroxylation and P450IA2 catalyzed relative rapid N-hydroxylation. In view of the capacity of PCN to decrease demethylation of methoxyphenoxazone (Table 1), a reaction that is efficiently catalyzed by P450IA2 (Fig. 1), it is not surprising that PCN did not elicit increases in AAF N-hydroxylation. This may be due to the capacity of PCN to repress P450IA2. Recently McManus *et al.* (42) found that PCN induced AAF 7-hydroxylation by about 2-fold in cultured rat hepatic cells, but hydroxylations at N or other carbons were not affected. DEX produced profound induction *in vivo*, but their data suggested that DEX was inducing several P450 isozymes.

BaP appeared to be attacked preferentially at carbon 3 by the PCN-induced isozyme(s) (Table 6) with lesser to negligible attack occurring at other carbon atoms. The 1-hydroxylated

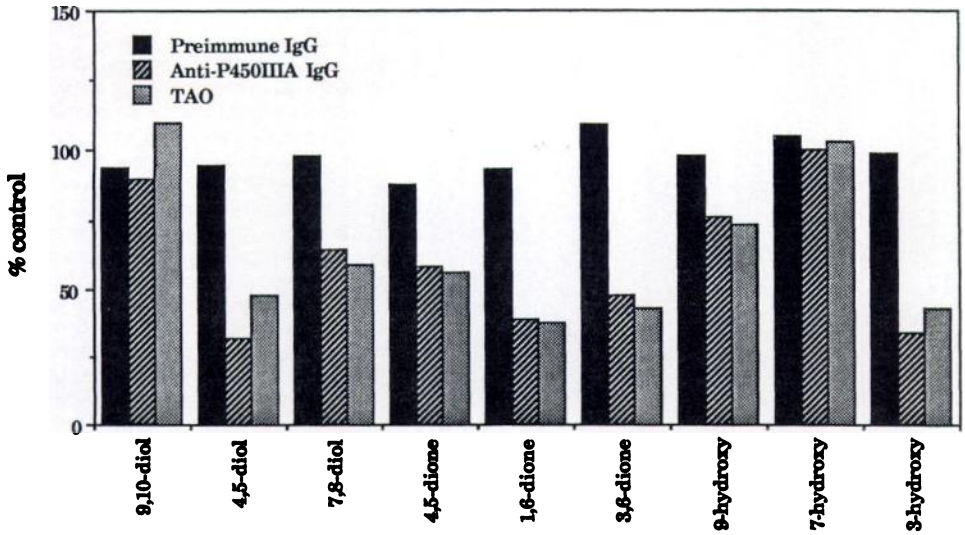

Fig. 5. Effects of preimune IgG, anti-P450IIIA1/2 IgG, and TAO on conversion of BaP to various hydroxylated metabolites. For other procedural details, see the legend to Fig. 4. Inhibition was not detected with microsomes from uninduced rats.

metabolite could not be detected but generation of small quantities of 1,6-quinone and inhibition of the generation of this metabolite by TAO or anti-P450IIIA1/2 antibody (Fig. 5) suggested some attack at the 1-carbon position. Reaction rates at carbon 7 were not increased and only minimally increased at carbon 9 by PCN pretreatment, suggesting that P450IIIA1 is an inefficient catalyst for 7- and 9-hydroxylation reactions. Because BaP is known to be a substrate for several P450 isozymes (Ref. 43 and references therein) and because of the results obtained with phenoxazone ethers (Table 1), it was of interest to directly compare freshly prepared microsomes from adult female rats treated with either highly purified PCN or DEX. Surprisingly, however, the results obtained (Table 6) showed primarily minor quantitative differences, with PCN exhibiting a slightly greater effect than DEX. An exception was in the generation of the 4,5-quinone which was decreased after pretreatment with DEX but slightly increased after PCN pretreatment.

Various other investigations (44–46) have reported marked increases in aryl hydrocarbon hydroxylase activity after PCN induction in immature or adult female rats. Inasmuch as this activity is primarily a reflection of BaP 3-hydroxylation, our results are in agreement but also demonstrate that other positions on the molecule do not appear to be attacked efficiently with the possible exception of carbon 6 (as evidenced from marked increases in quantities of 1,6- and 3,6-quinones) and, to a lesser extent, carbon 9.

An analysis of our data together with that reported in the literature by other investigators, provided a number of generalizations. One is that P450IIIA1 exhibits a relatively broad substrate specificity, its active site apparently capable of accommodating very large molecules including the highly bulky macrolide antibiotics. A relatively low degree of stereoselectivity with *R*- versus *S*-warfarin, relative lack of regioselectivity in the hydroxylation of AAF, and capacity to utilize a variety of chemicals as substrates also support this concept. Nevertheless, evidence for restricted substrate specificity was also observed. Comparisons of phenoxazone ethers, of attack at the β versus α face of the testosterone molecule, and regioselective attack at carbons 9 and 10 of the warfarin molecule provide examples of specificity.

A second generalization that can be drawn from the data is that testosterone and warfarin appear to be the most useful probes thus far encountered for the detection of members of the P450IIIA family in tissues containing only very small quantities of these isozymes. Some of the macrolide antibiotics, notably TAO, are reportedly specific substrates for this family (47), but current assays would probably not be sufficiently sensitive for measurements of the very low activities expected in most extrahepatic tissues, particularly during periods of early development. The data do suggest, however, that TAO may be very useful as an inhibitor probe for specific inhibition of P450IIIA-dependent reactions. Experiments were performed (Table 7) to further explore this idea (see also Figs. 3 and 4) and the results were promising, showing that TAO was more effective and at least as selective as inhibitor as polyclonal antibodies directed against P450IIIA. Thus, TAO should be very useful as an adjunct in probe analyses of tissues with low levels of this or closely related isozymes.

In terms of sensitivity, catecholestrogen formation, BaP 3-hydroxylation, AAF 7-hydroxylation, and benzyloxyresorufin

TABLE 7

Effects of TAO (0.1 mM) and polyclonal antibody directed against cytochrome P450IIIA1/2 (anti-P450IIIA1/2, 19 mg of IgG antibody protein/mg of microsomal protein) added as inhibitors *in vitro*

Concentrations selected followed preliminary range-finding experiments. Experiments were repeated twice with good agreement of percentages of inhibition. Values are percentages of activities observed in preparations containing an equal volume of TAO vehicle DMSO and an equal quantity of preimmune IgG, respectively. For reaction conditions, see the legends to Tables 1–3. TAO or IgG fractions were preincubated with all reaction mixture components (except substrate) at 37° for 5 min (TAO) or 25° for 15 min (IgG) prior to addition of substrate to initiate the reaction.

Reaction	Rat Hepatic Enzyme Source (Adult Females)	% Inhibition	
		TAO	Anti-P450IIIA
Debenzylation	PCN-induced	51	38
Debenzylation	Control	8	9
Warfarin 10-hydroxylation	PCN-induced	82	54
Warfarin 7-hydroxylation	Control	11	2
Testosterone 15β-hydroxylation	PCN-induced	77	41
Testosterone 16β-hydroxylation	PCN-induced	56	33
Testosterone 16α-hydroxylation	Control	5	4
Testosterone 2β-hydroxylation	PCN-induced	64	45

debenzylation are of proven value, but it is clear that these reactions can be catalyzed by several isozymes not included in the IIIA family. Warafin 10-hydroxylation appears highly specific for members of the IIIA family as discussed above and, as we have shown here, can also be very sensitive. Hydroxylations on the β face of testosterone exhibited both specificity and sensitivity. The 6β- and 16β-positions can be attacked by other P450 isozymes (48), but attack at the 2β- and 15β-positions appears to be specific for members of the P450IIIA family.

A large number of questions concerning the P450IIIA family remain to be answered. These include the number of isozymic forms that exist within the family (49), the modes of regulation, and substrate specificity of each form. It has been assumed that PCN induces only P450IIIA1, but a possibility exists that quantitatively minor P450 isozymes with higher substrate turnover numbers could be induced as well. An examination of the data suggests that this possibility seems somewhat remote in mature rats but of higher likelihood prenatally. Considerable additional research will be required to clarify issues pertaining to prenatal P450s.

The results of these investigations have provided a number of new and important findings. These include:

1. *Purified* PCN and *purified* DEX differ significantly in terms of their inducing properties. Purified PCN appears to be a more *specific* inducing agent. Commercially available PCN contains impurities with significant inducing properties.
2. P450IIIA1 will efficiently catalyze the biotransformation of two important model substrate carcinogens, AAF and BaP.
3. Data on the regiospecific monooxygenation of AAF and BaP are presented.
4. Data on the capacity of P450IIIA1 to catalyze the biotransformation of a series of phenoxazone ethers are presented.
5. The responses of fetal livers to the inducing effects of purified PCN or DEX differ from those of the adult female liver. Nevertheless, P450IIIA is induced in both.
6. TAO is a relatively specific inhibitor of P450IIIA1 and, because of the large and variable quantities of antibody often required for substratial inhibition, is probably more

reliably useful than polyclonal antibodies as an inhibitory probe.

7. Preliminary data indicating a decreased activity of P450IA2 after treatment with PCN or DEX are provided. These data are compatible with the idea that expression of IA2 may be repressed after treatment with these steroids.
8. Substantiation of previously reported results (with inducers of unknown purity) regarding the regio- and stereospecific biotransformation of testosterone and warfarin by P450IIIA, is presented.

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