Regulation of Rat Hepatic Cytochrome P-450: Age-Dependent Expression, Hormonal Imprinting, and Xenobiotic Inducibility of Sex-Specific Isoenzymes†

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ABSTRACT: The influence of age, sex, and hormonal status on the expression of eight rat hepatic cytochrome P-450 (P-450) isoenzymes was evaluated by both catalytic and immunochemical methods. The male specificity of P-450 2c(♂)/UT-A, the major microsomal steroid 16α-hydroxylase of uninduced rat liver [Waxman, D. J. (1984) *J. Biol. Chem. 259*, 15481–15490], was shown to reflect its ≥30-fold induction at puberty in male but not in female rats. The female specificity of P-450 2d(♀)/UT-I was shown to reflect its developmental induction in females. P-450 PB-2a/PCN-E was shown to mediate ≥85% of microsomal steroid 6β-hydroxylase activity; the male specificity of this P-450 largely reflects its developmental suppression in female rats. Neonatal gonadectomy and hormonal replacement experiments established that neonatal androgen "imprints" or programs the male rat for developmental induction of P-450 2c(♂)/UT-A, for maintenance of P-450 PB-2a/PCN-E, and for suppression of P-450 2d(♀)/UT-I, all of which occur in male rats at puberty. By contrast, the expressed levels of P-450 isoenzymes PB-1/PB-C, 3/UT-F, PB-4/PB-B, ISF-G, and βNF-B were mostly unaffected by the rats' age, sex, and hormonal status. Studies on the sex specificity of P-450 induction established that the response of these latter five isoenzymes to the P-450 inducers phenobarbital, β-naphthoflavone, pregnenolone-16α-carbonitrile, and isosafrole is qualitatively and quantitatively equivalent in females as in males. Although none of these agents effected an induction of P-450 2d(♀)/UT-I in males or an induction of P-450 2c(♂)/UT-A in females, pregnenolone-16α-carbonitrile administration led to a ≥70-fold induction of P-450 PB-2a/PCN-E in female rat liver, thereby abolishing the sex-specific expression of this steroid 6β-hydroxylase P-450. These studies establish that the multiple rat liver P-450s active in foreign compound metabolism are independently regulated by physiological factors and further serve to highlight the complex influences of foreign compound exposure on P-450-catalyzed steroid hormone hydroxylations in rat hepatic tissue.

Mammalian liver cytochrome P-450[1] is comprised of a family of heme protein isoenzymes that are active in the oxidative metabolism of structurally diverse lipophilic agents including drugs, pollutants, and other foreign compounds as well as physiological steroid hormones and fatty acids (Coon & Koop, 1983). Sex-related differences in P-450-mediated xenobiotic metabolism have been reported for several species, with the most striking differences observed in rats (Kato, 1974; Goble, 1975). Although the 10–30% lower level of total liver microsomal P-450 in female as compared to male rats is consistent with the slower metabolism of many drugs and environmental agents by the females, it does not explain the large sex-related differences [up to 20-fold, e.g., Kamataki et al. (1980)] observed for the rates of metabolism of some foreign compounds. Rather, these large sex-related differences probably reflect the contributions of sex-specific P-450 isoenzymes to overall microsomal metabolism. Support for this hypothesis is provided by recent immunochemical studies that demonstrate the existence in adult rat liver of both a male-specific P-450 isoenzyme [designated P-450 2c(♂)/UT-A in the present study] and a female-specific isoenzyme [designated P-450 2d(♀)/UT-I (Kamataki et al., 1983; Waxman, 1984)]. The markedly higher monooxygenase activity of P-450 2c(♂)/UT-A relative to P-450 2d(♀)/UT-I with many (though not all) of the monooxygenase substrates studied suggests that P-450 2c(♂)/UT-A contributes to the preferential metabolism of many xenobiotics by male rat liver. That other sex-dependent P-450s probably also contribute to this sex dependence of foreign compound metabolism is suggested by the sex difference for microsomal ethylmorphine metabolism (♂/♀ ~ 5; Chung et al., 1975; Nerland & Mannering, 1978), a difference that is not explained by the similar catalytic activities toward this substrate exhibited by P-450s 2c(♂)/UT-A and 2d(♀)/UT-I in purified, reconstituted systems (Waxman, 1984; Kamataki et al., 1983).

Adult rats are also known to metabolize steroid hormones in a sex-dependent fashion, suggesting that studies on those

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[1] Abbreviations: P-450, liver microsomal cytochrome P-450; KP_i, potassium phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; Na_2EDTA, disodium ethylenediaminetetraacetate; IgG, immunoglobulin G; 5α-reductase, NADPH:Δ^4-3-oxosteroid-5α-oxidoreductase; T, 17β-hydroxyandrost-4-en-3-one (testosterone); A, androst-4-ene-3,17-dione (androstenedione); 16α-CN-pregnenolone, pregnenolone-16α-carbonitrile; xα-OH-A, xβ-OH-A, etc., xα-hydroxyandrostenedione, xβ-hydroxyandrostenedione, etc., where *x* indicates the hydroxyl-substituted carbon atom. The term "sex-specific P-450" refers to a P-450 isoenzyme that is present at ≥10-fold higher levels in adult animals of one sex as compared to the other. The nomenclature used for individual forms or isoenzymes of P-450 is detailed under Experimental Procedures.

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P-450s active in steroid hormone hydroxylations may help to identify additional sex-dependent isoenzymes active in foreign compound metabolism. At least three classes of P-450-dependent steroid hormone hydroxylases have been distinguished on the basis of their sex specificity and responsiveness to gonadal hormon (Einarsson et al., 1973; Gustafsson & Stenberg, 1974). Enzymes in one group, which includes the male-specific, steroid 16α-hydroxylase recently identified as P-450 2c(δ)/UT-A (Waxman, 1984), are "imprinted" or programmed for their induction at puberty by exposure to androgen in the neonatal male rat. Enzymes in a second group, best exemplified by a steroid 6β-hydroxylase that is more active in male than in female rat liver, are reportedly reversibly inducible by androgens in adult rat liver. Included in the third group is a steroid 7α-hydroxylase that is present at moderately higher levels in female as compared to male rat liver. This enzyme appears to be regulated primarily by nongonadal factors.

In the present study we examine the relationship between these steroid hormone hydroxylase activities and the multiple P-450 isoenzymes purified in our laboratories from rat liver (Waxman & Walsh, 1982, 1983; Waxman, 1984; Guengerich et al., 1982a). The age and sex dependence of P-450 expression is reported for eight distinct isoenzymes, as is the identification of four of these isoenzymes as the predominant catalysts of microsomal metabolism of the C-19 steroid androstenedione at the 6β-, 7α-, 16α-, and 16β-positions, respectively. We also present experiments designed to evaluate the responsiveness of each of the developmentally regulated P-450s identified in this study to imprinting by neonatal androgen as well as studies on the sex dependence of P-450 isoenzyme induction in rat liver. A preliminary report on some of these studies has appeared (Dannan et al., 1984).

Experimental Procedures

Animals. Male and female rats of Sprague-Dawley descent ("CD") were purchased either from Charles River Breeding Laboratories (Wilmington, MA) or from Harlan Industries (Indianapolis, IN) and their liver microsomes prepared by standard methods (van der Hoeven & Coon, 1974). Adult male and female rats were induced with phenobarbital, β-naphthoflavone, 16α-CN-pregnenolone, or isosafrole by methods reported previously (Guengerich et al., 1982a) with all rats killed 24 h after the last treatment. Newborn rats of both sexes were gonadectomized on ice within 5 h of birth as described by Pfeiffer (1936). Testosterone propionate and estradiol benzoate were administered subcutaneously on days 1 and 3 of life to the birth gonadectomized animals [750 μg of androgen per animal per injection and dissolved at 25 mg/mL propylene glycol or 150 μg of estrogen per animal per injection and dissolved at 10 mg/mL propylene glycol] as indicated. Late castrations were performed at 5 weeks of age. All the gonadectomized animals were maintained under standard laboratory conditions until 10 weeks of age, at which time the animals were sacrificed and liver microsomes then prepared from individual rats.

Enzymatic Assays. Microsomal fractions [0.1 mg of microsomal protein/mL of 0.1 M KP_i (pH 7.4), 0.1 mM Na_2-EDTA, 1% (v/v) glycerol] were assayed for steroid hydroxylase activities by using [4-^{14}C]androstenedione, [4-^{14}C]testosterone, or [4-^{14}C]progesterone with the hydroxylated products resolved by thin-layer chromatography and then quantitated by methods described previously (Waxman, 1984; Waxman et al., 1983). In some cases, androstenedione metabolites were also analysed by high-performance liquid chromatography (Waxman, 1984). Microsomal 5α-reductase activities were determined in the same incubations used for steroid hydroxylase determinations by using [4-^{14}C]-androstenedione (reduced to [4-^{14}C]-5α-androstane-3,17-dione) as substrate. NADPH P-450 reductase activity was determined at 10 μg of microsomal protein per milliliter of 0.3 M KP_i (pH 7.7) and 0.1 mM EDTA at 30 °C by monitoring the reduction of cytochrome *c* (0.5 mg/mL) at 550 nm (ϵ = 21 mM^{-1} cm^{-1}) (Strobel & Dignam, 1978).

P-450 Isoenzymes. P-450 isoenzymes referred to in this study are designated by the combined nomenclatures used in our laboratories, as detailed below. Individual forms of P-450 were purified from rat hepatic microsomes as reported previously (Waxman, 1984; Waxman et al., 1983; Guengerich et al., 1982a). P-450 2d(♀)/UT-I was purified as we have described (Waxman, 1984) and also with the basic method of Kamataki et al. (1983); these preparations exhibited indistinguishable electrophoretic and immunochemical properties.

Equivalent forms of P-450 purified by other groups include the following: P-450 PB-2a/PCN-E = PCN-P-450 (Elshourbagy & Guzelian, 1980); P-450 2c(δ)/UT-A = P-450 RLM5 (Cheng & Schenkman, 1982) and P-450h (Ryan et al., 1984); P-450 2d(♀)/UT-I = P-450-female (Kamataki et al., 1983) and P-450i (Ryan et al., 1984); P-450 3/UT-F = P-450a (Ryan et al., 1982); P-450 PB-4/PB-B = P-450b (Ryan et al., 1982) and P-450 fraction C (West et al., 1979); P-450 PB-5/PB-D = P-450e (Ryan et al., 1982); P-450 βNF-B = P-450c (Ryan et al., 1982), P-448$_2$ (Imai, 1979), and P-450 MC-1 (Kuwahara et al., 1984); P-450 ISF-G = P-450d (Ryan et al., 1982), isosafrole P-450 (Fisher et al., 1981), P-448$_{HCB}$ (Goldstein et al., 1982), and P-450 MC-2 (Kuwahara et al., 1984). Purification of an isoenzyme corresponding to P-450 PB-1/PB-C (Guengerich et al., 1982a; Waxman & Walsh, 1983) has apparently not been reported by others. Although the biochemical properties and developmental dependence of an isoenzyme termed P-450-male (Kamataki et al., 1983; Maeda et al., 1984) suggest that this P-450 is equivalent to P-450 2c(δ)/UT-A, this conclusion should be viewed as tentative in the absence of information on the steroid hydroxylase activities of P-450-male.

Anti-P-450 Antibodies and Immunoquantitations. Antibodies to individual purified P-450s were raised in rabbits, and the IgG fractions were subsequently prepared by standard methods (Kaminsky et al., 1981). The isozymic specificities of these anti-P-450 antibodies were assessed both by Western blotting (see below) and by using an enzyme-linked immunosorbent assay (Waxman, 1984). In the cases where these techniques revealed a lack of monospecificity, the antisera (or IgG fractions) were further purified by cross-adsorption with covalently immobilized microsomes that were enriched with the cross-reactive antigen and, at the same time, contained low levels of the original immunogen. Microsomes were solubilized with 1.5% sodium cholate and 2% Lubrol PX and then coupled covalently to either Sepharose 4B (Thomas et al., 1979) or Reacti-Gel (Pierce Chemical Co., Rockford, IL) according to the manufacturers' recommendations. Antisera or IgG fractions were diluted in 20 mM KP_i (pH 7.4) containing 0.9% (w/v) NaCl and cycled through columns of such immunoadsorbants for 16 h at 22 °C. In cases where residual cross-reactivity was detected, the process was repeated with more immunoadsorbent until the desired specificity was achieved. In this manner, anti-P-450 2c(δ)/UT-A was passed through a column containing bound liver microsomes prepared from Aroclor 1254 treated female rats, anti-P-450 2d(♀)/UT-I was passed through a column containing bound liver micro-

Table I: Isozymic Specificity of Microsomal Androstenedione Hydroxylases: Antibody Inhibition Studies[a]

	IgG (mg)	% of control			
		6β-OH-A	7α-OH-A	16α-OH-A	16β-OH-A
PCN microsomes	0	(*8.20*)	(*0.18*)	(*1.33*)	(*0.53*)
+anti-PB-2a/PCN-E	0.15	46	30	104	64
	0.40	13*	9	72	52
+anti-3/UT-F	0.15	92	5*	94	71
+anti-ISF-G	0.15	74	69	71	107
ISF microsomes	0	(*5.58*)	(*0.43*)	(*0.47*)	(*1.43*)
+anti-PB-2a/PCN-E	0.40	4*	10	75	69
+anti-ISF-G	0.40	83	58	104	121
βNF microsomes	0	(*1.71*)	(*0.29*)	(*1.00*)	(*0.16*)
+anti-PB-2a/PCN-E	0.15	14*	47	102	76
+anti-3/UT-F	0.15	82	6*	92	82
+anti-ISF-G	0.15	72	58	97	77
+anti-βNF-B	0.15	73	107	95	70
+anti-2c(δ)/UT-A	0.01[b]	71	124	11*	90

[a] Liver microsomes prepared from either 16α-CN-pregnenolone- (PCN), isosafrole- (ISF), or β-naphthoflavone- (βNF) induced male rats were incubated with rabbit IgG fractions specific for the indicated P-450 isoenzymes and then assayed for androstenedione hydroxylase activities as described in Figure 1. Shown are the catalytic rates of the uninhibited microsomal incubations [values italicized and in parentheses; expressed as nmol of product min^{-1} (mg of microsomal protein)$^{-1}$] and the catalytic rates determined in the presence of added antibody and expressed as a percent of the uninhibited rates. Of the five anti-P-450 antibodies tested, only anti-P-450 PB-2a/PCN-E inhibited microsomal 6β-hydroxylase activity significantly, and as shown previously (Waxman, 1984) only anti-P-450 2c(δ)/UT-A inhibited microsomal 16α-hydroxylase activity (samples marked by asterisks). Although anti-P-450 3/UT-F selectively inhibited microsomal 7α-hydroxylase activity (asterisks), significant inhibition of this activity by anti-P-450 PB-2a/PCN-E (and, to a lesser extent, by anti-P-450 ISF-G) was also apparent. These latter inhibitions probably reflect the anti-P-450 3/UT-F activity endogenous to the anti-P-450 PB-2a/PCN-E (and anti-P-450 ISF-G) IgG preparations used in these experiments (see Experimental Procedures). [b] Affinity purified on an adult male microsome–Sepharose column after adsorption on adult female microsome–Sepharose (Waxman, 1984).

somes prepared from adult male rats, and anti-P-450 PB-4/PB-B and anti-P-450 βNF-B were passed through columns containing bound microsomes prepared from untreated and isosafrole-treated male rats, respectively. Although anti-P-450 PB-2a/PCN-E and anti-P-450 ISF-G were passed extensively through columns containing bound liver microsomes prepared from untreated adult female and phenobarbital-induced adult male rats, respectively, some residual cross-reactivity with P-450 3/UT-F (detectable by enzyme-linked immunosorbent assay but not on Western blots) remained in both antibody preparations. Immunoquantitations reported for P-450 PB-4/PB-B in the present study include the highly homologous and immunoreactive P-450 PB-5/PB-D. The cross-reactivity of the anti-P-450 ISF-G antibody with P-450 βNF-B did not interfere with the immunoquantitation of P-450 ISF-G by Western blotting as discussed previously (Dannan et al., 1983).

Quantitative estimates of individual P-450 isoenzyme levels in liver microsomal fractions were performed by Western blotting as described previously (Guengerich et al., 1982b) with the following modifications. First, a standard curve of purified P-450s was included on each nitrocellulose sheet. In addition, an internal standard consisting of a known amount of equine liver alcohol dehydrogenase (0.2 μg; Boehringer-Mannheim, Indianapolis, IN) was added to each sample or standard prior to electrophoresis, and a 1/300 dilution of rabbit anti-equine liver alcohol dehydrogenase antibody was mixed with the anti-P-450 antiserum prior to immunochemical staining. Thus, the stained nitrocellulose sheets typically contained two bands, one at M_r 43 000 (alcohol dehydrogenase) and one at M_r 48 000–56 000 (P-450). The intensities of both bands were estimated by densitometry (Guengerich et al., 1982b), and the ratios of the areas of the corresponding two peaks were used in all calculations. Inclusion of this internal standard helped to minimize variations in response between different gel lanes. Varying amounts of microsomal protein were assayed to identify the range of linear responses; the amounts of microsomal P-450 assayed were typically in the range of 0.1–3 pmol, depending upon the antigen and on the antibody. The dilution of the primary antiserum used for staining the nitrocellulose sheets was typically in the range of 1/50 to 1/400. Immunoquantitations were generally performed with microsomal samples prepared from individual animals and are expressed as nanomoles of P-450 isoenzyme per milligram of microsomal protein (mean ± SD for three to five individuals).

Results and Discussion

Isoenzyme Specificity of Microsomal Steroid Hydroxylations. Several rat liver P-450 isoenzymes have been shown to hydroxylate steroid hormones with a high degree of regio- and stereoselectivity, which contrasts to their broad and overlapping specificities for metabolism of xenobiotic substrates (Waxman et al., 1983; Wood et al., 1983; Cheng & Schenkman, 1984). The substrate specificity of these P-450s is most pronounced with the C-19 steroid androstenedione, which is selectively converted[2] to 7α-OH-A by P-450 3/UT-F, to 16α-OH-A by P-450 2c(δ)/UT-A, and to 16β-OH-A by P-450 PB-4/PB-B (Waxman, 1984). Antibody inhibition experiments were undertaken to evaluate whether the steroid hydroxylase activities catalyzed by unfractionated microsomal preparations can be ascribed to these or to other purified P-450 isoenzymes. Antibody specific for P-450 3/UT-F (see Experimental Procedures) was shown to inhibit selectively and quantitatively androstenedione 7α-hydroxylation catalyzed either by uninduced or by variously induced rat liver microsomes (Figure 1 and Table I). Antibody to P-450 PB-4/PB-B specifically inhibited androstenedione 16β-hydroxylase activity in PB-induced microsomes (Figure 1B), and antibody to P-450 2c(δ)/UT-A specifically inhibited microsomal 16α-hydroxylase activity (Figure 1A and Table I). These findings are consistent with the catalytic specificities exhibited by the corresponding purified P-450s and demonstrate that 7α-OH-A, 16β-OH-A, and 16α-OH-A are predominantly isoenzyme-specific microsomal metabolites of androstenedione (see Figure 4).

Although 6β-OH-A accounts for some 40–70% of the total monohydroxyandrostenediones formed by uninduced or var-

[2] For each isoenzyme, ≥85% of the total product formed corresponds to the indicated isomers.

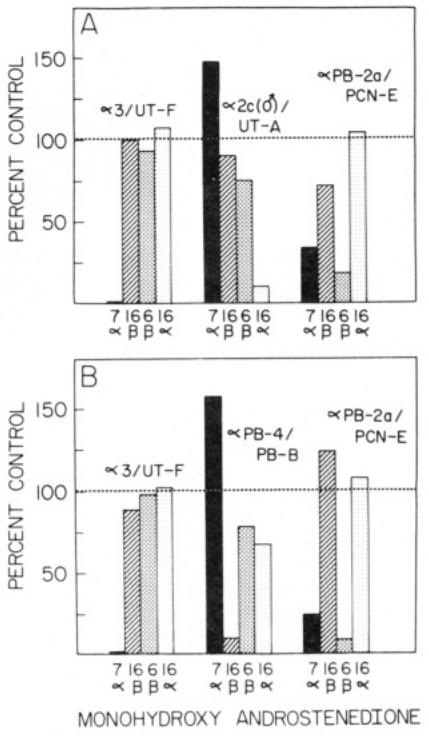

FIGURE 1: Antibody inhibition of microsomal androstenedione hydroxylase activities. Rabbit IgG fractions monospecific for P-450s 3/UT-F (0.15 mg of IgG), 2c(δ)/UT-A (0.01 mg of affinity-purified antibody; see Table I, footnote *b*), PB-2a/PCN-E (0.15 mg of IgG, panel A; 0.4 mg of IgG, panel B), or PB-4/PB-B (1.0 mg of IgG) were incubated for 30 min at 22 °C in a final volume of 375 μL with adult male rat liver microsomes (40 μg) prepared from either uninduced (panel A) or phenobarbital-induced (panel B) animals. Androstenedione hydroxylase activities were then measured (see Experimental Procedures) and the results expressed as percents of the uninhibited controls (horizontal dashes lines) for each of the four major monohydroxyandrostenedione products formed. Control catalytic rates (see Table IV for representative values) were largely unaffected (≤20% inhibition of activity) by incubation with 0.15–1.0 mg of preimmune IgG. Stimulation of microsomal 7α-hydroxylase activity by antibody to P-450s 2c(δ)/UT-A and PB-4/PB-B may result from a more effective competition of P-450 3/UT-F for the rate-limiting (Miwa et al., 1978) microsomal NADPH P-450 reductase.

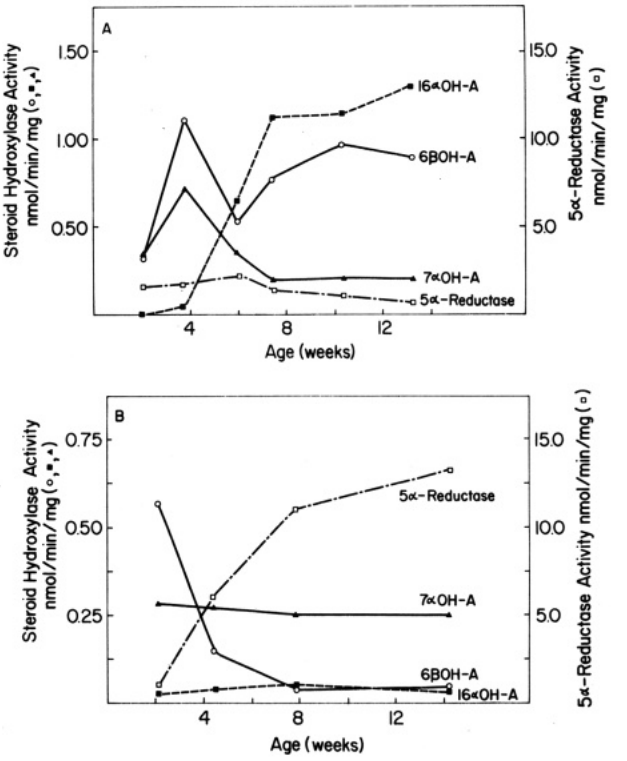

FIGURE 2: Age-dependent expression of androstenedione hydroxylase activities in male (panel A) and female (panel B) rat liver. Pooled liver microsomes were prepared from uninduced male and female rats 2–14 weeks of age with 10 animals included in the 2-week-old groups and five animals in each of the others. Androstenedione hydroxylase and Δ^4-steroid 5α-reductase activities were determined as described under Experimental Procedures.

iously induced rat liver microsomes (see, e.g., Table IV), the P-450 isoenzyme catalyzing this 6β-hydroxylation has not been identified. Although several P-450s, including P-450 βNF-B, P-450 ISF-G, and P-450 2c(δ)/UT-A, exhibit some 6β-hydroxylase activity in reconstituted systems [turnover of 0.2–0.6 nmol of 6β-OH-A min^{-1} (nmol of P-450)$^{-1}$], antibody inhibition experiments indicated that none of these P-450s catalyzes a major fraction of microsomal androstenedione 6β-hydroxylation (Table I). By contrast, rabbit antibody raised to purified P-450 PB-2a/PCN-E effectively inhibited microsomal androstenedione 6β-hydroxylation (Figure 1 and Table I). Larger amounts of anti-P-450 PB-2a/PCN-E were required for complete inhibition of 6β-hydroxylation catalyzed by liver microsomes prepared from rats pretreated with phenobarbital, 16α-CN-pregnenolone, or isosafrole, agents that induce both 6β-hydroxylase activity and P-450 PB-2a/PCN-E levels several-fold (see Tables IV and V). Although the anti-PB-2a/PCN-E antibody used in these experiments also inhibited microsomal androstenedione 7α-hydroxylase activity, this effect probably reflects contamination of the antibody by anti-P-450 3/UT-F as detected with enzyme-linked immunosorbent methods. The low androstenedione 6β-hydroxylase activity of purified P-450 PB-2a/PCN-E (turnover of ~0.2 min^{-1} P-450^{-1}; data not shown) is consistent with the selective loss of androstenedione 6β-hydroxylase activity observed upon cholate solubilization of liver microsomes [85% loss of 6β-hydroxylase as compared to <10% loss of 16α-hydroxylase activity; data not shown; also see Shiverick & Neims (1979)] and suggests that purified P-450 PB-2a/PCN-E is at least partially inactivated during isolation. The low ethylmorphine demethylase and low warfarin 10-hydroxylase activities exhibited by purified P–450 PB-2a/PCN-E are consistent with this possibility (Elshourbagy & Guzelian, 1980; Guengerich et al., 1982a).

Antibody inhibition studies were also performed to ascertain the isozymic contributions to microsomal metabolism of testosterone, progesterone, and 17β-estradiol. The results obtained (data not shown) indicate that the microsomal metabolites 2α-OH-testosterone and 2α-OH-progesterone are predominantly formed by P-450 2c(δ)/UT-A, 6β-OH-testosterone and 6β-OH-progesterone by P-450 PB-2a/PCN-E, 7α-OH-testosterone by P-450 3/UT-F, and 16β-OH-testosterone by P-450 PB-4/PB-B, in agreement with the catalytic specificities exhibited by the purified P-450s in reconstituted systems (Waxman, 1984).

Developmental Expression of P-450 Isoenzymes. P-450-dependent steroid hydroxylase activities are known to undergo distinct developmental changes in male rat liver [e.g., Jacobson & Kuntzman (1969) and Pasleau et al. (1981)]. This developmental regulation was examined at the level of individual P-450 isoenzymes in the present study by monitoring isozyme-specific monohydroxyandrostenedione metabolites (Figure 2) and also by immunoquantitation of eight distinct P-450 isoenzymes in liver microsomes prepared from male and female rats aged 2–14 weeks (Figure 3). Three P-450 isoenzymes were thus shown to be developmentally regulated in a sex-specific fashion. The male specificity of P-450 2c(δ)/UT-A and its corresponding microsomal androstenedione

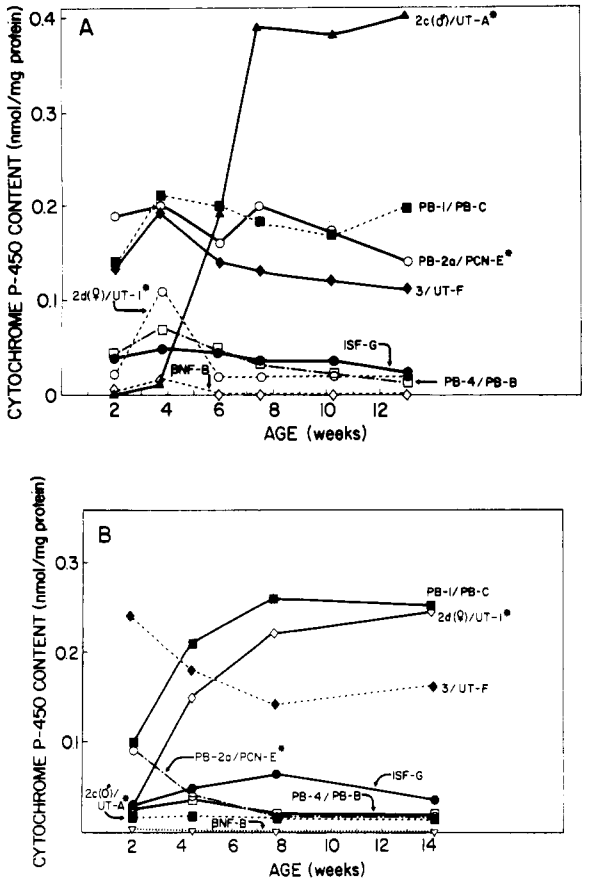

FIGURE 3: Age-dependent expression of individual P-450 isoenzymes in male (panel A) and female (panel B) rat liver. Liver microsomes from male and female rats aged 2–14 weeks (see Figure 2) were analyzed for eight individual P-450 isoenzymes by Western blotting as described under Experimental Procedures. In general, values graphed at or below 0.02–0.03 nmol/mg were below the limits of detection in these experiments (e.g., the levels of P-450 2d (♀)/UT-I in male rats at all ages other than 4 weeks). P-450 isoenzymes exhibiting pronounced sex specificities are marked with asterisks (*).

16α-hydroxylase activity (Waxman, 1984) are now shown to reflect a >30-fold induction of this isoenzyme at puberty in male but not female rat liver. P-450 PB-2a/PCN-E and its corresponding microsomal 6β-hydroxylase activity were also found to be male specific in adult rat liver (♂/♀ > 20). However, the sex specificity of this P-450 largely reflects its developmental reduction in maturing females and not its developmental induction in males (Figures 2B and 3B). Finally, a third sex-specific P-450, P-450 2d(♀)/UT-I, was shown to be developmentally induced in female rats, reaching its adult level by 8 weeks of age. A similar developmental pattern was seen for Δ^4-steroid 5α-reductase, another female-specific liver microsomal enzyme (♀/♂ > 10) (Figure 2B). Although P-450 2d(♀)/UT-I is female specific in adult rat liver (♀/♂ > 20), it is also expressed at significant levels (40–50% of the adult female levels) in 4-week-old males (Figure 3A).

Other P-450 isoenzymes examined in this study exhibited much less marked age and sex dependencies. P-450 3/UT-F and its corresponding androstenedione 7α-hydroxylase activity were present at 50–100% higher levels in adult females as compared to adult males. This enzyme was shown to peak at 4 weeks in male rats and then decrease at puberty by ~50%. P-450 3/UT-F did not exhibit as striking an age dependence in female rat liver. P-450 PB-1/PB-C was found to be present at similar levels in male and female rat liver, with a significant increase in enzyme levels (50% to >100%) occurring from 2 to 4 weeks of age in both sexes. Consistent with this observation, MacDonald et al. (1981) have reported a corresponding age-dependent increase in the microsomal warfarin 7-hydroxylase activity characteristic of purified P-450 PB-1/PB-C (Guengerich et al., 1982a; Waxman & Walsh, 1983). Three other P-450 isoenzymes, termed P-450 PB-4/PB-B, P-450 βNF-B, and P-450 ISF-G, were found to be present at low levels in uninduced animals of both sexes and did not exhibit marked age dependencies.

Neonatal Imprinting of P-450 Isoenzymes. The sex specificity and developmental regulation of P-450s 2c(♂)/UT-A, PB-2a/PCN-E, and 2d(♀)/UT-I suggest that one or more of these isoenzymes may correspond to the neonatally "imprinted" or programmed P-450-dependent steroid hydroxylase activities found in rat liver (Einarsson et al., 1973; Gustafsson et al., 1983). The influence of neonatal hormone secretions on the postpubertal expression of these three P-450 isoenzymes was therefore investigated. Newborn rats of both sexes were either untreated, gonadectomized within 5 h of birth, birth gonadectomized and subsequently administered single doses of testosterone propionate or estradiol benzoate on days 1 and 3 of life, or gonadectomized at 5 weeks of age. At 10 weeks all the rats were killed and their liver microsomes subsequently analyzed both for individual P-450 isoenzymes by Western blot analysis and for their associated microsomal steroid hydroxylase activities. Birth castration, but not late castration, was found to abolish the expression of both P-450 2c(♂)/UT-A and P-450 PB-2a/PCN-E in the 10-week-old males (Table II). This effect could be reversed by two single injections of testosterone propionate during the first 3 days of life (column 5 vs. 4), thus demonstrating that the expression of these two P-450s in adult rat liver is neonatally imprinted. Although the neonatal hormone thus administered did not fully restore adult enzyme levels (columns 5 vs. 2), ≥75% restoration of the imprinted enzyme levels (defined as those exhibited by the late castrated animals; column 3) was achieved. That these imprinted levels were 20–40% lower than the adult enzyme levels (column 2) is consistent with the proposed reversible stimulation of neonatally imprinted liver steroid hydroxylases by postpubertal gonadal secretions (Gustafsson & Stenberg, 1974). Birth gonadectomized females did not express these two male-specific P-450s (Table III, column 4), demonstrating that the imprinted state results from a positive programming by neonatal androgen and not from the absence of negative programming by neonatal estrogen. A small induction of P-450 2c(♂)/UT-A was, however, seen in rats ovariectomized at 5 weeks of age (column 3).

Although estrogen exposure is not required for basal level expression of P-450 2d(♀)/UT-I in adult females [Table III, column 3 vs. 2; also see Kamataki et al. (1983)], neonatal estrogen exposure and, to a greater extent, adult estrogen exposure stimulate expression of this female-specific P-450 isoenzyme. Birth gonadectomy of male rats resulted in a low but significant level of expression of P-450 2d(♀)/UT-I in the adult animals (Table II). This effect was abolished by neonatal administration of testosterone propionate and was also not observed upon castration at 5 weeks of age. That the level of expression of P-450 2d(♀)/UT-I in the birth castrated adult males (0.08 nmol/mg; Table II) was nearly equivalent to that found in untreated 4-week-old males (0.11 nmol/mg; Figure 3A) indicates that the suppression of this P-450 in male rats at puberty is neonatally imprinted. Thus, the female specifity of P-450 2d(♀)/UT-I reflects both a stimulatory effect of estrogen in the females and a neonatally programmed suppression of P-450 2d(♀)/UT-I in male rats after 4 weeks of age.

Table II: Influence of Castration on P-450 Isoenzymes in Male Rat Liver[a]

	nmol of P-450 isoenzyme/mg or nmol of product min^{-1} mg^{-1}			
	untreated	late castration	birth castration	birth castration/TP
P-450 2c(♂)/UT-A[b]	0.37 ± 0.02	0.21 ± 0.03	0.02 ± 0.02	0.18 ± 0.05
16α-OH-A	2.12 ± 0.18	1.21 ± 0.20	0.12 ± 0.03	0.92 ± 0.15
2α-OH-T	1.08 ± 0.27	0.61 ± 0.02	0.08 ± 0.01	0.54 ± 0.03
P-450 PB-2a/PCN-E	0.23 ± 0.02	0.24 ± 0.05	<0.02	0.17 ± 0.01
6β-OH-A	1.89 ± 0.28	1.39 ± 0.29	0.12 ± 0.03	1.50 ± 0.21
6β-OH-T	1.78 ± 0.28	1.27 ± 0.08	0.12 ± 0.03	1.64 ± 0.03
P-450 2d(♀)/UT-I	<0.006	<0.006	0.08 ± 0.05	<0.006
P-450 3/UT-F	0.11 ± 0.01	0.11 ± 0.01	0.13 ± 0.02	0.13 ± 0.01
7α-OH-A	0.22 ± 0.04	0.22 ± 0.04	0.30 ± 0.05	0.33 ± 0.10
7α-OH-T	0.21 ± 0.03	0.20 ± 0.03	0.37 ± 0.03	0.40 ± 0.12
P-450 PB-1/PB-C	0.35 ± 0.07	0.31 ± 0.10	0.40 ± 0.05	0.39 ± 0.03
NADPH P-450 reductase	360 ± 19	331 ± 25	304 ± 36	322 ± 29

[a] Liver microsomes were prepared from 10-week-old male rats that were untreated, castrated at 5 weeks of age (late castrated), or castrated at birth. One group of birth-castrated rats was administered testosterone propionate on days 1 and 3 of life (TP). Individual P-450s were immunoquantitated by Western blotting with the results expressed as nmol of P-450/mg (mean ± SD for three to five individual animals). Microsomal steroid hydroxylase and NADPH P-450 reductase activities were determined as described under Experimental Procedures and are expressed as nmol of product min^{-1} mg^{-1} (mean ± SD for three to four independent determinations, each performed with microsomes pooled from three to five individual animals). [b] Similar results were obtained for the relative catalytic activities of P-450 2c(♂)/UT-A in these four groups of rats when the microsomes were assayed for testosterone 16α-hydroxylase and for estradiol 2-hydroxylase activities.

Table III: Influence of Ovariectomy on P-450 Isoenzymes in Female Rat Liver[a]

	nmol of P-450 isoenzyme/mg or nmol of product min^{-1} mg^{-1}			
	untreated	late ovariectomy	birth ovariectomy	birth ovariectomy/EB
P-450 2c(♂)/UT-A	<0.02	0.05 ± 0.03	<0.01	<0.01
16α-OH-A	0.05 ± 0.02	0.17 ± 0.04	0.06 ± 0.02	0.04 ± 0.02
2α-OH-T	0.04 ± 0.02	0.11 ± 0.02	0.08 ± 0.03	0.06[b]
P-450 PB-2a/PCN-E	<0.01	<0.01	<0.01	<0.01
6β-OH-A	0.10 ± 0.04	0.09 ± 0.03	0.08 ± 0.02	0.06 ± 0.02
6β-OH-T	0.11 ± 0.03	0.08 ± 0.01	0.09 ± 0.02	0.09
P-450 2d(♀)/UT-I	0.23 ± 0.05	0.11 ± 0.06	0.08 ± 0.03	0.12 ± 0.04
P-450 3/UT-F	0.17 ± 0.01	0.14 ± 0.02	0.16 ± 0.01	0.13 ± 0.01
7α-OH-A	0.54 ± 0.11	0.42 ± 0.16	0.31 ± 0.02	0.25 ± 0.03
7α-OH-T	0.54 ± 0.04	0.39 ± 0.04	0.36 ± 0.06	0.14
P-450 PB-1/PB-C	0.37 ± 0.03	0.35 ± 0.04	0.23 ± 0.03	0.23 ± 0.01
NADPH P-450 reductase	267 ± 20	277 ± 28	260 ± 30	289 ± 41

[a] Liver microsomes were prepared from 10-week-old female rats that were untreated, ovariectomized at 5 weeks of age (late ovariectomy), or ovariectomized at birth. One group of birth-ovariectomized rats was administered estradiol benzoate on days 1 and 3 of life (EB). Individual P-450s were immunoquantitated and catalytic rates determined as described in Table II. [b] Catalytic activities shown for this group are for a single determination when testosterone was used as substrate.

Although birth castration did not elevate P-450 3/UT-F or its associated steroid 7α-hydroxylase activities to the levels characteristic of female rat liver, birth ovariectomy reduced steroid 7α-hydroxylases activities by 50% to the levels characteristic of male rat liver[3] (Table II and III). A somewhat smaller decrease in activity was observed upon ovariectomy at 5 weeks of age, suggesting a positive imprinting by prepubertal estrogen. Estradiol benzote injections on days 1 and 3 of life did not, however, restore normal female levels in the adult animals but, in fact, suppressed 7α-hydroxylase activity even further. This suggests that the imprinting process may require hormonal exposure over a longer period of time or possibly at a later age. The imprinting of the P-450-dependent 7α-hydroxylase active on 5α-androstane-3α,17β-diol [probably corresponding to P-450 3/UT-F] is not complete until 14–30 days after birth and may involve nongonadal factors (Gustafsson & Stenberg, 1974; Einarsson et al., 1973). Gonadectomy and hormonal injections were shown to have relatively small effects on the levels of P-450 PB-1/PB-C and NADPH P-450 reductase, two enzymes that do not exhibit marked sex dependencies (Tables II and III).

Sex Dependence of P-450 Isoenzyme Induction by Xenobiotics. The influence of sex on P-450 induction in adult rat liver was examined by assaying for four isoenzyme-specific microsomal androstenedione metabolites and by immunoquantitation of eight individual P-450 isoenzymes in liver microsomes prepared from both induced and uninduced adult rats of both sexes (Tables IV and V). The four monooxygenase inducers examined were shown to suppress P-450 2c(♂)/UT-A and its associated microsomal steroid 16α-hydroxylase activity in adult male rat liver, in agreement with our earlier observations (Guengerich et al., 1982a; Waxman, 1984). Up to 90% suppression of this isoenzyme occurs upon administration of certain polybrominated biphenyl congeners (Dannan et al., 1983). Although a small but measurable induction of the P-450 2c(♂)/UT-A characteristic microsomal androstenedione 16α-hydroxylase (Table IV) and testosterone 2α-hydroxylase activities (data not shown) was observed in adult females administered the synthetic steroid 16α-CN-pregnenolone, a corresponding induction of P-450 2c(♂)/UT-A was not detected (Table V).

In contrast to the suppression of P-450 2c(♂)/UT-A by

[3] A corresponding decrease in P-450 3/UT-F levels upon birth castration was not apparent, suggesting that factors other than the polypeptide levels may regulate this steroid 7α-hydroxylase P-450.

Table IV: Sex Dependence of Xenobiotic Induction: Androstenedione Hydroxylases[a]

liver microsomes	NADPH-P-450 reductase	nmol of product min^{-1} mg^{-1} 16α-OH-A	6β-OH-A	7α-OH-A	16β-OH-A
adult male rat	385 ± 26	2.40	2.19	0.23	0.35
+phenobarbital	521 ± 55	1.93	6.31	0.45	8.43
+β-naphthoflavone	313 ± 23	1.01	1.56	0.31	0.17
+16α-CN-pregnenolone	427 ± 34	1.55	7.91	0.28	0.58
+isosafrole	628 ± 46	0.71	7.34	0.54	2.00
adult female rat	256 ± 8	0.05	0.10	0.54	0.13
+phenobarbital	483 ± 30	0.48	0.52	1.32	5.02
+β-naphthoflavone	301 ± 22	0.06	0.19	1.01	0.07
+16α-CN-pregnenolone	614 ± 28	0.31	1.57	0.65	0.82
+isosafrole	632 ± 43	0.19	0.48	1.13	1.09

[a] Adult rats of both sexes either were untreated or were induced with one of the four agents shown (three to five animals per group). Catalytic activities were determined for the isolated liver microsomes (see Experimental Procedures). Androstenedione hydroxylase activities are expressed as averages of duplicate determinations.

Table V: Sex Dependence of Xenobiotic Induction: P-450 Isoenzymes[a]

liver microsomes	P-450 isoenzyme (nmol of P-450 isoenzyme/mg of microsomal protein) 2c(♂)/UT-A	PB-2a/ PCN-E	2d(♀)/UT-I	3/UT-F	PB-4/PB-B	PB-1/PB-C	βNF-B	ISF-G
adult male rat	0.30 ± 0.04	0.23 ± 0.02	<0.01	0.11 ± 0.01	0.08 ± 0.02	0.35 ± 0.07	<0.03	0.09 ± 0.01
+phenobarbital	0.16	0.66	<0.01	0.22 ± 0.02	1.50 ± 0.21	0.88 ± 0.10	<0.03	0.08 ± 0.01
+β-naphthoflavone	0.19	0.11	<0.01	0.15 ± 0.01	0.07	0.23 ± 0.01	1.22 ± 0.24	0.54 ± 0.06
+16α-CN-pregnenolone	0.19	1.07	<0.01	0.06 ± 0.01	0.22	0.20 ± 0.01	<0.03	0.08 ± 0.01
+isosafrole	0.06	0.34	<0.02	0.15	0.38	0.23	0.62	1.04
adult female rat	<0.02	<0.01	0.22 ± 0.03	0.17 ± 0.01	0.07 ± 0.02	0.37 ± 0.03	<0.03	0.09 ± 0.01
+phenobarbital	<0.02	0.19	0.22 ± 0.01	0.30 ± 0.06	1.07 ± 0.05	0.77 ± 0.03	<0.03	0.09 ± 0.01
+β-naphthoflavone	<0.02	<0.01	0.27 ± 0.06	0.28 ± 0.05	0.06	0.27 ± 0.04	1.10 ± 0.38	0.64 ± 0.05
+16α-CN-pregnenolone	<0.02	0.70	0.32 ± 0.04	0.12 ± 0.02	0.23	0.23 ± 0.02	<0.03	0.08 ± 0.01
+isosafrole	<0.02	0.13	0.20	0.27	0.34	0.14	0.65	1.31

[a] Individual P-450 isoenzymes were immunoquantitated by Western blotting using the same microsomal samples analyzed in Table IV. Values are expressed as mean ± SD for determinations performed with individual microsomes from three to five animals or, in cases when a single value is tabulated, as the averages of duplicate determinations performed with pooled microsomes from the three to five individual animals.

classical monooxygenase inducers, P-450 PB-2a/PCN-E was induced in male rats 3-fold by phenobarbital and ~5-fold by 16α-CN-pregnenolone administration (Table V). This 5-fold induction of immunoreactive P-450 PB-2a/PCN-E was, however, accompanied by only a 3–4-fold induction of its associated microsomal 6β-hydroxylase activity (Table IV), suggesting that some component other than the heme protein catalyst, possibly NADPH P-450 reductase, had become rate limiting upon induction with 16α-CN-pregnenolone. The sex-specificity characteristic of P-450 PB-2a/PCN-E in uninduced adult rats (♂/♀ > 20) was markedly reduced upon administration of phenobarbital (♂/♀ = 3.5) or 16α-CN-pregnenolone (♂/♀ = 1.5) largely as a consequence of the much greater induction (up to ≥70-fold) of this P-450 isoenzyme in female rats (Table V). This latter observation provides an explanation for the apparent discrepancy between the 4-fold induction of P-450 PB-2a/PCN-E by 16α-CN-pregnenolone obtained by Guengerich et al. (1982a) with adult male rats and the >20-fold induction reported by Heuman et al. (1982) with adult females.

Although microsomal 6β-hydroxylase activity was induced in the xenobiotic-treated females in parallel to P-450 PB-2a/PCN-E in the current study, the specific 6β-hydroxylase activity of the induced female microsomes was only ~25–30% of that of the correspondingly induced male microsomes [e.g., 7.91/1.07 = 7.4 nmol of 6β-OH-A min^{-1} (nmol of microsomal PB-2a/PCN-E)$^{-1}$ for the 16α-CN-pregnenolone-induced males vs. 1.57/0.70 = 2.2 nmol of 6β-OH-A min^{-1} (nmol of microsomal PB-2a/PCN-E)$^{-1}$ for the corresponding females (Tables IV and V)]. Possible explanations for this lower specific 6β-hydroxylase activity in the females would include (1) higher apoprotein levels, (2) less effective competition for NADPH P-450 reductase, and (3) induction of an immunochemically cross-reactive but catalytically less active form of P-450 PB-2a/PCN-E in the female rats.

The level of expression and female specificity of P-450 2d(♀)/UT-I was largely unaffected by xenobiotic administration, with an induction of this isoenzyme (~50% increase) seen only upon 16α-CN-pregnenolone administration (Table V). P-450 3/UT-F and its corresponding microsomal androstenedione 7α-hydroxylase activity were induced up to 2-fold or 3-fold in both sexes by a variety of agents, with the highest activities found in phenobarbital- or isosafrole-induced adult females. Sex differences were not observed for the phenobarbital induction of either P-450 PB-1/PB-C (~2-fold induction) or P-450 PB-4/PB-B and its corresponding 16β-hydroxylase activity (>20-fold induction). Isosafrole induced the latter isoenzyme while suppressing the former isoenzyme in both sexes. The two principal polycyclic-inducible P-450s, βNF-B and ISF-G, were induced to similar levels in both sexes upon administration of either β-naphthoflavone or isosafrole (Table V).

Conclusions

In summary, studies on the developmental regulation of rat hepatic cytochrome P-450 have identified three isoenzymes, termed P-450 2c(♂)/UT-A, P-450 2d(♀)/UT-I, and P-450 PB-2a/PCN-E, which exhibit marked age and sex dependencies. The male specificity of P-450 2c(♂)/UT-A, the major microsomal steroid 16α-hydroxylase and steroid 2α-hydroxylase of rat liver (Waxman, 1984), was shown to result from its developmental induction at puberty in male but not in female rats. The female specificity of P-450 2d(♀)/UT-I, which exhibits structural and immunochemical homology to P-450 2c(♂)/UT-A (Waxman, 1984), was shown to reflect its developmental induction in females. This female-specific

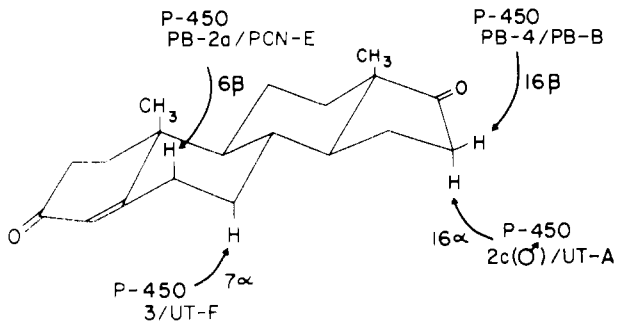

FIGURE 4: Regio- and stereoselectivity of the four major microsomal androstenedione hydroxylase P-450s of rat liver.

P-450 was, however, found to be present at significant levels in immature (4-week-old) male rats, in agreement with the recent findings of Maeda et al. (1984). P-450 PB-2a/PCN-E was shown to mediate the majority (>85%) of microsomal steroid 6β-hydroxylase activity by means of antibody inhibition studies (Figure 4). Previous workers have reported that this 6β-hydroxylase activity is present at from 3-fold up to 20-fold higher levels in adult male as compared to adult female rat liver [e.g., Einarsson et al. (1973) and Levin et al. (1975)]. In the present study this male specificity was shown to result from a developmental reduction of P-450 PB-2a/PCN-E and its corresponding steroid 6β-hydroxylase activity in maturing female rats.

Studies on the hormonal regulation of these sex-dependent P-450 isoenzymes have shown that neonatal androgen secretion plays an essential role in imprinting the age and sex dependence of their expression. Thus, neonatal androgen programs for the development induction of P-450 2c(♂)/UT-A in male rat liver, for the maintenance of P-450 PB-2a/PCN-E in maturing males, and for the decrease in P-450 2d(♀)/UT-I that occurs in male rats after 4 weeks of age. Changes in the corresponding microsomal steroid hydroxylase activities with development and with hormonal status are primarily due to alterations in enzyme levels rather than due to modulation of their catalytic activities. The previously studied neonatal programming of sex-specific, P-450-dependent microsomal steroid hydroxylase activities (Colby, 1980; Gustafsson et al., 1983) can thus be defined in terms of well-characterized P-450 isoenzymes, setting the stage for more detailed studies on the mechanisms of their hormonal regulation.

The induction of eight distinct P-450 isoenzymes by various foreign compounds was examined in both males and females to ascertain whether important sex differences also characterize the induction of rat hepatic P-450s by xenobiotics. The induction of P-450 PB-4/PB-B by phenobarbital (>20-fold induction) and by isosafrole (~5-fold), of P-450s PB-1/PB-C and 3/UT-F by phenobarbital (~2-fold), and of P-450s βNF-B and ISF-G by both β-naphthoflavone and isosafrole (up to >40-fold) was shown to proceed in a manner that is qualitatively and quantitatively equivalent in both sexes. Xenobiotic administration did not lead to induction of P-450 2d (♀)/UT-I in males or of P-450 2c(♂)/UT-A in females. In fact, the latter isoenzyme and its corresponding steroid 16α-hydroxylase activity were suppressed in livers of male rats pretreated with several monooxygenase inducers. Xenobiotic administration did, however, lead to a marked induction of the male-specific P-450 PB-2a/PCN-E. This occurred not only in male rats (4–5-fold induction by 16α-CN-pregnenolone) but also in female rats (≥70-fold induction), thereby abolishing the sex-specific expression of this steroid hormone 6β-hydroxylase P-450. Taken together, these observations suggest that classic P-450 inducers can interfere with steroid hormone metabolism in several ways, including (1) induction of new hydroxylation pathways (e.g., >25-fold induction of the P-450 PB-4/PB-B mediated microsomal androstenedione 16β-hydroxylation by phenobarbital) or enhancement of existing pathways (e.g., 3–4-fold induction of the P-450 PB-2a/PCN-E mediated steroid 6β-hydroxylation by 16α-CN-pregnenolone), (2) suppression of hormonally regulated hydroxylation pathways [e.g., 60–70% decrease in the P-450 2c(♂)/UT-A mediated steroid 16α-hydroxylation upon β-naphthoflavone or isosafrole administration], and (3) induction of hydroxylation pathways otherwise subject to strict endocrine control (e.g., ~15-fold induction by 16α-CN-pregnenolone of the P-450 PB-2a/PCN-E mediated 6β-hydroxylase activity in adult female rats).

Sex-related differences in rat hepatic xenobiotic metabolism have been explained in part by the distinctive catalytic specificities exhibited by P-450 2c(♂)/UT-A and by P-450 2d-(♀)/UT-I in reconstituted systems, with the latter isoenzyme exhibiting lower monooxygenase activity with many of the foreign compound substrates tested (Kamataki et al., 1983; Waxman, 1984). These observations do not, however, explain the marked male specificity of microsomal ethylmorphine *N*-demethylase activity (♂/♀ ~ 5; Nerland & Mannering, 1978) since purified P-450 2d(♀)/UT-I exhibits good catalytic activity with this monoxygenase substrate. Rather, the present studies suggest that the male-specific metabolism of ethylmorphine may reflect the major contribution made by the male-specific P-450 PB-2a/PCN-E to microsomal ethylmorphine metabolism (Elshourbagy & Guzelian, 1980). Thus, at least three sex-specific, P-450-dependent steroid hydroxylase P-450s are likely to contribute to the sex dependence of foreign compound metabolism characteristic of rat liver.

Registry No. Cytochrome P-450, 9035-51-2; phenobarbital, 50-06-6; β-naphthoflavone, 6051-87-2; pregnenolone-16α-carbonitrile, 1434-54-4; isosafrole, 120-58-1; androstenedione 7α-hydroxylase, 9042-09-5; steroid 16β-hydroxylase, 37359-58-3; steroid 16α-hydroxylase, 37364-16-2; steroid 6β-hydroxylase, 9075-83-6; steroid 5α-reductase, 9036-43-5; testosterone, 58-22-0; estradiol, 50-28-2.

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Quantitation of *S*-Adenosylmethionine Decarboxylase Protein†

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ABSTRACT: A method for the specific labeling of the active site of *S*-adenosylmethionine decarboxylase was developed. The method consisted of incubating cell extracts with ^{3}H-decarboxylated *S*-adenosylmethionine and sodium cyanoborohydride in the presence of a spermidine synthase inhibitor. Under these conditions, *S*-adenosylmethionine decarboxylase was labeled specifically and stoichiometrically. This procedure was used (a) to establish that the subunit molecular weight of *S*-adenosylmethionine decarboxylase from rat liver, prostate, and psoas and from mouse SV-3T3 cells was 32 000, (b) to titrate the number of active molecules of *S*-adenosylmethionine decarboxylase in various cell extracts, and (c) to provide a high specific activity labeled preparation of *S*-adenosylmethionine decarboxylase for use in radioimmunoassay of this enzyme. Competitive radioimmunoassays using this labeled antigen had a sensitivity such that 3 fmol (0.1 ng) of enzyme protein could be quantitated. The rapid loss of *S*-adenosylmethionine decarboxylase which occurred when SV-3T3 cells were exposed to exogenous polyamines was shown to be due to a rapid decline in the amount of enzyme protein measured both by titration of the active site and by radioimmunoassay.

The biosynthesis and interconversion of polyamines in mammalian cells are highly regulated processes, and several of the enzymes involved in this pathway exhibit remarkable fluctuations in activity under a wide range of physiological conditions (Jänne et al., 1978; Pegg & McCann, 1982; Tabor & Tabor, 1984a). These enzymes [ornithine decarboxylase, spermidine/spermine N^1-acetyltransferase, and *S*-adenosylmethionine decarboxylase (AdoMetDC)] are present in very small amounts even after maximal induction (Pegg et al., 1982). Therefore, methods for the quantitative estimation of the amount of protein, which are needed to investigate the mechanism by which changes in enzyme activity are brought about, have been difficult to develop. Recently, advances in the understanding of the regulation of ornithine decarboxylase have been made by the use of a technique in which the enzyme is labeled stoichiometrically by reaction with radioactive α-(difluoromethyl)ornithine (DFMO) (Pritchard et al., 1981). Such labeling has been used to titrate the number of enzyme molecules present in cell extracts, to identify the protein after

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