Regioselective Progesterone Hydroxylation Catalyzed by Eleven Rat Hepatic Cytochrome P-450 Isozymes

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ABSTRACT: Quantitative high-pressure liquid chromatographic assays were developed that separate progesterone and 17 authentic monohydroxylated derivatives. The assays were utilized to investigate the hydroxylation of progesterone by 11 purified rat hepatic cytochrome P-450 isozymes and 8 different rat hepatic microsomal preparations. In a reconstituted system, progesterone was most efficiently metabolized by cytochrome P-450h followed by P-450g and P-450b. Seven different monohydroxylated progesterone metabolites were identified. 16α-Hydroxyprogesterone, formed by 8 of the 11 isozymes, was the only detectable metabolite formed by cytochromes P-450b and P-450e. 2α-Hydroxyprogesterone was formed almost exclusively by cytochrome P-450h, and 6α-hydroxyprogesterone and 7α-hydroxyprogesterone were only formed by P-450a. 6β-Hydroxylation of progesterone was catalyzed by four isozymes with cytochrome P-450g being the most efficient, and 15α-hydroxyprogesterone was formed as a minor metabolite by cytochromes P-450g, P-450h, and P-450i. None of the isozymes catalyzed 17α-hydroxylation of progesterone, and only cytochrome P-450k had detectable 21-hydroxylase activity. 16α-Hydroxylation catalyzed by cytochrome P-450b was inhibited in the presence of dilauroylphosphatidylcholine (1.6–80 μM), while this phospholipid either stimulated (up to 3-fold) or had no effect on the metabolism of progesterone by the other purified isozymes. Results of microsomal metabolism in conjunction with antibody inhibition experiments indicated that cytochromes P-450a and P-450h were the sole 7α- and 2α-hydroxylases, respectively, and that P-450k or an immunochemically related isozyme contributed >80% of the 21-hydroxylase activity observed in microsomes from phenobarbital-induced rats.

The term cytochrome P-450[1] refers to a family of enzymes with a characteristic reduced carbon monoxide absorption maximum near 450 nm. The enzymes function as monooxygenases by transferring one oxygen atom (formally an oxene) from molecular oxygen to a substrate while the other atom of molecular oxygen is reduced to water. Reducing equivalents are shuttled to the hepatic enzymes from NADPH by way of NADPH–cytochrome P-450 reductase (White & Coon, 1980; Guengerich & Macdonald, 1984). Individual isozymes of this ubiquitous enzyme superfamily find their tissue levels affected by organic modifiers, age, sex, strain, species, and genetic makeup.

The monooxygenase activity associated with cytochromes P-450 has been shown to be mandatory for many biochemical processes including xenobiotic degradation and steroid hormone biosynthesis (Conney, 1967; Engel, 1973; Hall, 1985). In general, xenobiotic degradation has been associated primarily with hepatic isozymes and steroid biosynthesis associated primarily with the isozymes located in the adrenal gland and gonads. Though steroids have been shown to be substrates for the hepatic isozymes, the major documented pathways of androgen and progesterone elimination are through reduction and conjugation (Fotherby & James, 1972). It is not known whether steroid hydroxylations catalyzed by the hepatic isozymes lead to biologically important products or are just coincidences due to the inherent broad substrate selectivity of these enzymes. Studies that characterize the role of each hepatic cytochrome P-450 isozyme in the regioselective metabolism of steroids will provide valuable information regarding these questions.

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Experimental Procedures

Instrumentation. Chemical ionization mass spectra were obtained on a Finnigan 1015 GC/MS[2] equipped with a Finnigan 9500 GC and a Finnigan 6000 data system with Revision I software (Finnigan Instrument Corp., Sunnyvale, CA).

Electron impact mass spectra (m/z 10–700) were obtained on a Vg ZAB-2F with a Vg 2025 data system. Samples were introduced through the direct insertion probe inlet.

Proton NMR spectra were obtained with a Varian XL-400 spectrometer operated at 400 MHz in the Fourier transform mode. Tetramethylsilane was the internal reference, and the solvent was $CDCl_3$.

Chemicals. Progesterone and [4-^{14}C]progesterone were obtained from Sigma Chemical Co. (St. Louis, MO) and New England Nuclear (Boston, MA), respectively. 6α-, 11α-, 12α-, and 14α-OHP were a gift of the Steroid Reference Collection, MCR, London, U.K. 15α-OHP was a gift of Dr. Harold Karns (Upjohn, Kalamazoo, MI); 11β-hydroxytestosterone and 16α-, 17α-, 18-, and 19-OHP were purchased from Sigma;

[1] According to a recently recommended nomenclature system for cytochromes P-450 (Nebert et al., 1987), P-450a is encoded by rat gene IIA1, P-450b by IIB1, P-450c by IA1, P-450d by IA2, P-450e by IIB2, P-450f by IIC7, P-450j by IIE1, and P-450k by IIC6. Cytochromes P-450g, P-450h, and P-450i have not been classified although the total amino acid sequence of P-450h has recently been obtained from its cDNA (Yoshioka et al., 1987).

[2] Abbreviations: HPLC, high-pressure liquid chromatography; GC/MS, gas chromatography/mass spectroscopy; TLC, thin-layer chromatography; ODS, octadecylsilane; P-450a, P-450b, etc., cytochrome P-450a, cytochrome P-450b, etc.; 16α-OHP, 17α-OHP, etc., 16α-hydroxyprogesterone, 17α-hydroxyprogesterone, etc.; NADPH 5α-reductase, 3-oxo-5α-steroid:NADP$^+$ Δ^4-oxidoreductase (EC 1.3.1.22); SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

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2α-, 6β-, 11β-, 20α-, 20β-, and 21-OHP, 6- and 16-ketoprogesterone, 6-dehydroprogesterone, and 16α,17α-epoxyprogesterone were purchased from Steraloids, Inc. (Wilton, NH).

Dilauroyl-*sn*-glycero-3-phosphorylcholine, purchased from Calbiochem (San Diego, CA), was prepared in water and sonicated immediately before use. Other chemicals were obtained from Sigma, and organic solvents were from Burdick and Jackson Laboratories, Inc. (Muskegon, MI), with the exception of HPLC-grade tetrahydrofuran, which was purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ).

Synthesis. (*A*) *2β-Hydroxyprogesterone* was synthesized according to the method of Rao et al. (1963). The crystalline product had suitable chromatographic properties in that while it comigrated with only 2α-OHP in HPLC system I (see below), it separated from 2α-OHP in the TLC system described by Einarsson and Gustafsson (1973) (3/1 benzene/ethyl acetate) and was obtained as the trimethylsilyl ether with GC/MS. It also resolved from all other available monohydroxyprogesterones. Electron impact mass spectrometry: m/z 330 (M^+), 312 ($M^+ - OH_2$), 286 ($M^+ - C_2H_4O$). NMR analysis was consistent with an A-ring β-hydroxylated steroid.

(*B*) *15β-Hydroxyprogesterone.* 15β-OHP was prepared by epimerizing 15α-OHP according to the Mitzunobu reaction (Mitzunobu, 1981). 15α-OHP (24 mg, 0.073 mmol) and triphenylphosphine (42 mg, 0.16 mmol) were dissolved in 1 mL of a 4/1 solution of benzene/tetrahydrofuran and stirred at room temperature. To this solution, benzoic acid (9.8 mg, 0.08 mmol) in 0.1 mL of benzene was added, followed by diethyl azodicarboxylate (13.9 mg, 0.08 mmol) in 0.1 mL of benzene. The solution, after refluxing for 5 h, was stirred at room temperature overnight. The solvents were evaporated, and the residue was redissolved in 95/5 ethyl acetate/acetone, filtered, and then purified by silica gel chromatography (30 × 1 cm). The product, 15β-(benzyloxy)progesterone, had an R_f of 0.20 (silica gel TLC, 95/5 ethyl acetate/acetone). The solvent was evaporated under a stream of nitrogen, and the product was redissolved in 0.5 mL of MeOH and 0.1 mL of 1.0 N NaOH. After this solution was stirred at room temperature for 48 h, 1 mL of H_2O was added and the pH adjusted to 8.5. The solution was extracted with methylene chloride (2 × 2 mL) and dried over anhydrous sodium sulfate, and the methylene chloride was evaporated.

The resulting product contained 15α-OHP and 15β-OHP. 15β-OHP was isolated by HPLC (system I) and showed suitable chromatographic properties; the electron impact mass spectrum was identical with published spectra (Zaretskii et al., 1966): m/z 231 ($M^+ - C_5H_7O_2$), 269 ($M^+ - H_2O - CH_3CO$), 312 ($M^+ - H_2O$), 330 (M^+). The ratio of m/z 330 to 312 was 0.25 ± 0.13, which is consistent with the literature value of 0.20. This is in agreement with the axial stereochemistry of the 15β-OH group. The fragment at m/z 231 is due to loss of the C-15, C-16, and C-17 carbons of the steroid D ring and the substituents associated with these carbons. NMR analysis showed a C-18 signal at 0.88 ppm, indicating that the D ring hydroxy group must be β-oriented due to the downfield shift.

(*C*) *16β-Hydroxyprogesterone.* 16β-OHP was synthesized with liver microsomes from male rats according to the method of Siekmann et al. (1980). Incubations consisted of potassium phosphate buffer, pH 7.4 (0.01 M), nicotinamide (0.02 M), magnesium chloride (0.01 M), sucrose (0.25 M), rat liver microsomes (2 mg/mL), and 16α,17α-epoxyprogesterone (120 μM) in a total volume of 10 mL. All incubations were carried out at 30 °C for 30 min. The reaction was stopped, extracted with methylene chloride (25 mL), washed with 0.01 N NaOH (2 × 10 mL) and H_2O (1 × 10 mL), and then dried over anhydrous sodium sulfate. 16β-OHP was isolated by HPLC (system I, see below) and showed chromatographic properties consistent with its identification. Methane chemical ionization mass spectrometry on the trimethylsilyl ether derivative of the metabolite showed a parent ion of m/z 403, the electron impact mass spectrum was consistent with that reported, and the product did not comigrate with other D-ring hydroxyprogesterones.

Microsomes, Enzymes, and Antibody Preparations. Long–Evans rats (3- and 8-weeks old) were obtained from Blue Spruce Farms (Altamont, NY). Adult female rats were treated with dexamethasone (100 mg kg^{-1} day^{-1} in corn oil, ip) for 3 days, and animals were killed on the fourth day. All other treatments of rats and preparation of microsomes were as previously described (Bandiera et al., 1986). Cytochromes P-450a–P-450k were purified to apparent homogeneity as previously reported (Ryan et al., 1982, 1984a, 1985; Bandiera et al., 1986). All of the purified cytochrome P-450 isozymes were homogeneous on SDS–polyacrylamide gels.

Total cytochrome P-450 was determined by the method of Omura and Sato (1964) in potassium phosphate buffer, pH 7.4 (0.05 M), with 20% glycerol. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard.

NADPH–cytochrome P-450 reductase was purified from phenobarbital-treated rats to a specific activity of 40 000 units/mg of protein by a combination of the methods of Dignam and Strobel (1975) and Yasukochi and Masters (1976). One unit of reductase catalyzes the reduction of 1 nmol of cytochrome *c* per minute at 22 °C in 0.3 M potassium phosphate buffer (pH 7.7) containing 0.1 mM EDTA and 0.1 mM NADPH.

Production, purification, and characterization of polyclonal and monoclonal antibodies used in this study were as previously described (Thomas et al., 1981, 1984; Reik et al., 1982, 1985; Bandiera et al., 1986). The monoclonal antibody C8 is specific for P-450c. Monoclonal antibody B50 is specific for P-450b in ELISA and on immunoblots but inhibits completely the catalytic activity of P-450b and partially inhibits the catalytic activity of P-450e in reconstituted systems (Reik et al., 1985). The polyclonal antibodies were made monospecific for the antigen of immunization as described with two exceptions. Anti-P-450d also recognizes P-450c, and anti-P-450k recognizes the immunochemically related family of isozymes that includes P-450f, P-450g, P-450h, P-450i, and P-450k.

Enzyme Assays. Incubations with hepatic microsomes contained cytochrome P-450 (0.2 nmol), NADPH (1 μmol), magnesium chloride (3 μmol), sucrose (10 μmol), potassium phosphate buffer, pH 7.4 (50 μmol), and progesterone (250 nmol in 20 μL of methanol) in a total volume of 1 mL and were agitated for 5 min at 37 °C following addition of substrate.

Incubations with purified cytochrome P-450 isozymes, unless otherwise stated, contained cytochrome P-450 (0.02–0.10 nmol), NADPH–cytochrome P-450 reductase (1500 units, 0.4 nmol), dilauroylphosphatidylcholine (10 μg), NADPH (0.5 μmol), magnesium chloride (3 μmol), potassium phosphate buffer, pH 7.4 (50 μmol), and progesterone (50 nmol in 20 μL of methanol) in a 1-mL final volume. Samples were incubated at 37 °C for 5 min. Catalytic activities of all enzyme preparations were determined under conditions in which metabolism was proportional to cytochrome P-450 concentration and time of incubation. NADPH–cytochrome P-450 reductase

concentration was saturating and dilauroylphosphatidylcholine concentration optimal. Substrate concentrations above 65 μM were found to be inhibitory for the two isozymes investigated (P-450b and P-450g), so all incubations contained 50 μM progesterone, a concentration near the limit of solubility of progesterone in water at 37 °C (42 μM) (Bischoff & Pilhorn, 1948). All reactions were terminated by addition of 1 nmol of internal standard (11β-hydroxytestosterone) in 50 μL of methanol followed immediately by 6 mL of methylene chloride. After mixing and centrifugation of the samples, the organic phases were evaporated under a stream of nitrogen, and the residues were dissolved in 200 μL of acetonitrile and analyzed by HPLC.

HPLC. Analysis of metabolic reactions was performed on a Perkin-Elmer Series 4 liquid chromatograph, equipped with an ISS-100 autosampler and an LC-95 UV spectrophotometer (254 nm).

Separation of progesterone, its metabolites, and the internal standard, 11β-hydroxytestosterone, was achieved with an IBM 5-μm, 25-cm, ODS column preceded by a 5-cm guard column handpacked with ODS. The column was eluted with water/acetonitrile/tetrahydrofuran/methanol under the following conditions (system I): 3 min isocratic at 85/6/4/5; 25 min with a 0.5 convex gradient to 68/11/8/13; 10 min with a linear gradient to 57.5/14.5/11/17; 10 min with a linear gradient to 15/57/11/17 followed by a 5-min ramp to 3/90/4/3. In instances when further resolution of 16α-, 15β-, and 7α-OHP was needed, a second system (system II) was used as follows: 3 min isocratic 85/13/1/1; 15 min with a 0.5 gradient to 68/30/1/1; 10 min with a linear gradient to 57.5/14.5/11/17 with the column and LC setup above. For optimum resolution of all standards, columns with greater than 75000 theoretical plates per meter were necessary. Resolution was not affected up to 50 nmol of each metabolite per injection. All chromatographic separations were performed at room temperature.

Metabolites were quantitated by comparing peak height of metabolites with internal standard peak height. A standard curve of metabolite peak height vs that of 11β-hydroxytestosterone showed linearity at all concentrations tested (1–67 nmol). All monohydroxyprogesterones and the internal standard were shown to have >99% extractability under the conditions of this assay as determined from comparing ratios of known amounts before and after incubation and extraction, as well as counting radioactivity remaining in the aqueous phase after metabolism and extraction of [4-^{14}C]progesterone. When [4-^{14}C]progesterone was used as substrate, peaks corresponding to UV absorbance at 254 nm gave essentially identical values as the radioactive metabolites quantitated by scintillation spectroscopy.

Separation of 2α- and 2β-Hydroxyprogesterone. Since 2α- and 2β-OHP were found to comigrate regardless of the mobile-phase composition, it was necessary to derivatize these metabolites to achieve separation. This was accomplished by collecting the 2-OHP peak off HPLC system I and determining the total amount of 2α- and 2β-OHP formed. The mobile phase was evaporated under a stream of nitrogen, and the hydroxyprogesterones were acetylated by the addition of benzene (1 mL), 0.05 N triethylamine in benzene (100 μL), and acetic anhydride (15 μL) to the reaction vessel, which was tightly capped and heated at 60 °C for 24 h. The solvents were then evaporated under a stream of nitrogen, and >99% conversion to 2α- and 2β-acetoxyprogesterone was achieved according to HPLC analysis. The residue was dissolved in 150 μL of acetonitrile, and the products were separated with an isocratic reverse-phase HPLC system (system III). The mobile-phase composition was 55% water, 18% acetonitrile, 25% methanol, and 2% tetrahydrofuran. The ratio of 2α-acetoxy- (retention time 41 min) to 2β-acetoxyprogesterone (retention time 44 min) was determined at a flow rate of 2.5 mL/min.

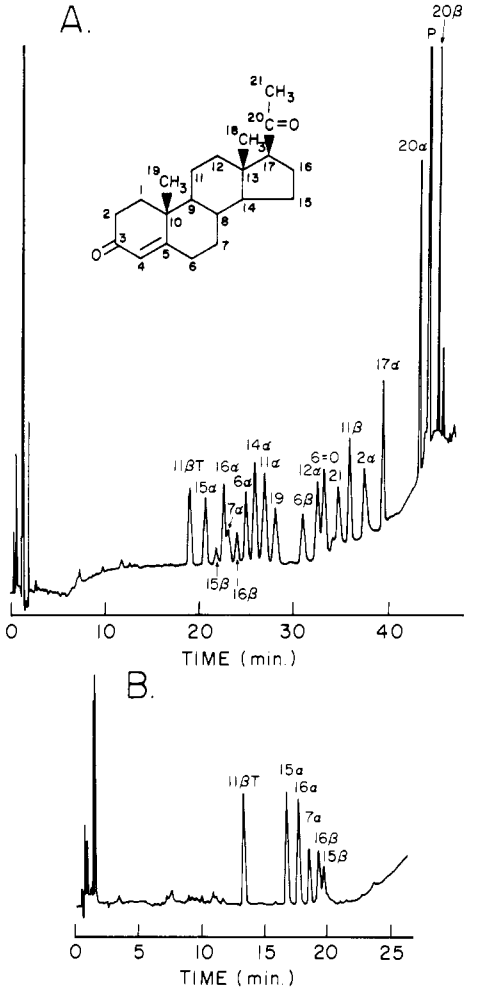

FIGURE 1: HPLC chromatograms of progesterone and available reference compounds. (A) Profile of progesterone, 18 known or potential progesterone metabolites, and the internal standard 11β-hydroxytestosterone (11βT) eluted by system I. (B) Profile of five potential progesterone metabolites and the internal standard 11β-hydroxytestosterone eluted by system II. Details are given under Experimental Procedures. P is the abbreviation for progesterone, and with the exception of 6=O (which represents 6-ketoprogesterone) and 11βT (which represents 11β-hydroxytestosterone), the remaining abbreviations represent monohydroxy derivatives of the parent steroid.

RESULTS

Metabolite Identification and Chromatographic Conditions. The hydroxylated products of progesterone metabolism were identified by comigration with authentic standards. 2β-, 15β-, and 16β-OHP were unavailable for purchase and were thus synthesized (see Experimental Procedures). 7α-OHP was tentatively identified on the basis of its having the correct molecular weight for a monohydroxyprogesterone, its comparative mobility on TLC relative to other monohydroxyprogesterone standards being similar to that reported for authentic 7α-OHP (Gustafsson & Lisboa, 1970), and its being the major metabolite of P-450a, the isozyme responsible for 7α-hydroxylation of testosterone and androstenedione (Wood et al., 1983).

A reverse-phase HPLC assay that separates the potential metabolites of progesterone is shown in Figure 1A. By utilization of the HPLC optimization method first described by

Table I: Metabolism of Progesterone by Purified Rat Liver Cytochrome P-450 Isozymes[a]

	nmol min^{-1} (nmol of P-450)$^{-1}$										
metabolite	P-450a	P-450b	P-450c	P-450d	P-450e	P-450f	P-450g	P-450h	P-450i	P-450j	P-450k
2α	–	–	–	–	–	0.3	–	26.4	–	–	–
2β	–	–	–	–	–	–	–	–	–	–	–
6α	0.4	–	–	–	–	–	–	–	–	–	–
6β	–	–	5.1	3.6	–	–	10.2	1.7	–	–	–
7α	7.3	–	–	–	–	–	–	–	–	–	–
11α	–	–	–	–	–	–	–	–	–	–	–
11β	–	–	–	–	–	–	–	–	–	–	–
12α	–	–	–	–	–	–	–	–	–	–	–
14α	–	–	–	–	–	–	–	–	–	–	–
15α	–	–	–	–	–	–	0.6	0.2	0.5	–	–
15β	–	–	–	–	–	–	–	–	–	–	–
16α	–	17.1	2.8	0.9	3.7	0.6	12.2	20.4	–	–	1.0
16β	–	–	–	–	–	–	–	–	–	–	–
17α	–	–	–	–	–	–	–	–	–	–	–
19	–	–	–	–	–	–	–	–	–	–	–
20α	–	–	–	–	–	–	–	–	–	–	–
20β	–	–	–	–	–	–	–	–	–	–	–
21	–	–	–	–	–	–	–	–	–	–	0.9
total	7.7	17.1	7.9	4.5	3.7	0.9	23.0	48.7	0.5	–	1.9

[a] Dashes indicate that no significant amount of product was observed [<0.1 nmol min^{-1} (nmol of cytochrome P-450)$^{-1}$]. Incubations and analytical procedures were as described under Experimental Procedures.

Glajch et al. (1980) with a modification that lends itself to four solvent gradient optimization (to be published elsewhere), base line or near base line resolution was achieved with all but two pairs of metabolites. The 7α- and 16α-OHP pair was easily separated in another system (Figure 1B), but separation of 2α- and 2β-OHP required a more complex approach as detailed under Experimental Procedures.

Oxidation of Progesterone Catalyzed by Purified Cytochrome P-450 Isozymes. Metabolism of progesterone to monohydroxylated products is reported in Table I. The progesterone molecule has 24 nonequivalent carbon–hydrogen bonds suitable for cytochrome P-450 catalyzed hydroxylation. The 11 isozymes investigated formed a total of 7 monohydroxyprogesterones of which all except 7α-OHP comigrated with authentic standards. Metabolite profiles for each isozyme showed marked regioselectivity and a distinct pattern. Cytochrome P-450a metabolized progesterone predominantly (>94%) to one metabolite, 7α-OHP. The major phenobarbital-inducible isozymes, P-450b and P-450e, formed exclusively (>99%) one metabolite, 16α-OHP. And as previously observed with other substrates, P-450b was a more efficient catalyst than P-450e, though they differ in only 13 of 491 amino acids (Fujii-Kuriyama et al., 1982; Yuan et al., 1983). Another phenobarbital-inducible isozyme, P-450k, in addition to functioning as a 16α-hydroxylase, albeit inefficient as compared to P-450b and P-450e, was the sole 21-hydroxylase observed. The major polycyclic aromatic hydrocarbon inducible isozymes, P-450c and P-450d, both catalyzed the formation of 6β-OHP and 16α-OHP with 6β-OHP being formed more expeditiously. Cytochrome P-450d catalyzed the metabolism of progesterone at the 6β- and 16α-positions at 70% and 32%, respectively, of the efficiency of P-450c. Of the noninducible enzymes, only the male-specific proteins, P-450g and P-450h, had respectable activities. Cytochrome P-450h formed 2α-OHP and 16α-OHP with high turnover rates as well as minor amounts of 15α-OHP and 6β-OHP. Cytochrome P-450g hydroxylated the 6β- and 16α-position with approximately equal efficiency and the 15α-position much less effectively. Cytochromes P-450g and P-450h were also the only isozymes where a significant number of polar secondary metabolites were observed. The noninducible female-specific protein P-450i, a protein shown to have isozyme-specific activity for 15β-hydroxylation of 5α-androstane-3α,17β-diol 3,17-disulfate (Gustafsson & Ingelman-Sundberg, 1975; Ryan et al., 1984b), catalyzed the formation of 15α-OHP with low but detectable activity. Cytochrome P-450f showed low activity for 16α- and 2α-hydroxylation of progesterone, and no activity was detectable with the ethanol-inducible isozyme, P-450j.

The integrity of each purified isozyme's observed catalytic profile can be evaluated through a comparison of potential contributions from trace amounts of other isozymes that could contaminate the enzyme preparation. Cytochrome P-450a, for example, is not contaminated with any of the other isozymes used in this study because the metabolite profile associated with P-450a is unique to itself—P-450a is the only known 6α- and 7α-hydroxylase. Cytochromes P-450b and P-450e, which form exclusively 16α-OHP, cannot be contaminated with any of the other six isozymes that catalyze 16α-hydroxylation, as the other isozymes catalyze metabolism of progesterone at additional positions at the same order of magnitude as 16α-hydroxylation. If P-450b and P-450e were contaminated with these enzymes, additional metabolites would have been observed.

The integrity of the 6β- and 16α-hydroxylation associated with P-450c and P-450d can be monitored by monospecific inhibitory antibodies. For example, if P-450c or P-450d were contaminated with 15% or 5% of P-450b, respectively, a monospecific inhibitory antibody to P-450c or P-450d would not inhibit 16α-hydroxylation. As monospecific inhibitory antibodies against P-450c and P-450d inhibited all detectable catalytic activity associated with these enzymes (Table II), the integrity of their metabolite profile is confirmed. In a similar fashion, P-450c, P-450d, P-450f, and P-450g were all shown to be homogeneous because the catalytic activity was abolished by the homologous monospecific inhibitory antibody (Table II).

A concern of this investigation was to determine if 6β-OHP was in fact a metabolite formed by P-450h. This concern arose because P-450h catalyzed the metabolism of testosterone (Ryan et al., 1984a) to 16α-hydroxytestosterone, 2α-hydroxytestosterone, and a negligible amount of 6β-hydroxytestosterone. Although P-450c and P-450d have been shown to have 6β-hydroxylase activity, they could not have contributed significantly to the 6β-hydroxylase activity associated with P-450h because (1) P-450h was purified from

Table II: Antibody Inhibition of Progesterone Metabolism Catalyzed by Purified P-450 Isozymes[a]

P-450 isozyme	antibody	mg of IgG/nmol of P-450	nmol min^{-1} (nmol of P-450)$^{-1}$			
			2α-OH	6β-OH	16α-OH	21-OH
c	control		–	2.1	1.3	–
c	anti-P-450c (C8)	0.2	–	<0.1	<0.1	–
d	control		–	1.9	0.6	–
d	anti-P-450(–c)	10.0	–	<0.1	<0.1	–
f	control		0.2	–	0.5	–
f	anti-P-450f	10.0	<0.1	–	<0.1	–
g	control		–	7.0	10.1	–
g	anti-P-450g	10.0	–	<0.1	0.9	–
h	control		29.4	2.0	27.8	–
h	anti-P-450g	10.0	27.9	2.0	26.1	–
k	control		–	–	0.6	0.4
k	anti-P-450b (B50)	0.2	–	–	0.7	0.5

[a] Cytochrome P-450 isozymes were mixed with either rabbit control IgG or monospecific anti-P-450 IgG. Cytochrome P-450 and IgG were incubated 5 min at room temperature, reductase and phospholipid were added, and incubations were continued for 5 min. Ten-minute incubations with progesterone and analytical procedures were as described under Experimental Procedures. Monospecific polyclonal or monoclonal (C8 and B50) antibodies have been previously characterized as described under Experimental Procedures. Dashes indicate that no significant amount of product was observed (<0.1 nmol min^{-1} nmol^{-1}). Data represent the mean of duplicates.

control male rats, which have been shown to have very low amounts of P-450c and P-450d (Thomas et al., 1983), and (2) the amount of P-450c or P-450d contamination required to 6β-hydroxylate progesterone at the rate of 1.7 nmol min^{-1} nmol^{-1} would be 33% and 50% for P-450c and P-450d, respectively. This amount of impurity would be easily observed by SDS gel electrophoresis. The only other isozyme investigated that could contribute to the 6β-hydroxylase activity of P-450h is P-450g, which is also a male-specific protein with considerable 6β-hydroxylase activity. However, monospecific anti-P-450g did not significantly inhibit the hydroxylation of progesterone by P-450h (Table II). Finally, a monoclonal antibody (H80) specific for P-450h (Thomas et al., 1987) gave 80% inhibition of all metabolites formed by purified P-450h (data not shown).

The 16α-hydroxylase activity associated with P-450k, a phenobarbital-inducible enzyme, was confirmed with antibody against the other two phenobarbital-induced 16α-hydroxylases, as the monoclonal antibody B50 did not inhibit the 16α-hydroxylase activity associated with P-450k. It must be noted that these experiments cannot eliminate the possibility of catalytic contribution from isozymes that have not been isolated by this laboratory. However, any such isozyme is not detectable in SDS gels or by N-terminal sequence analysis and would also have to react with the antibodies used in this study.

Dependence of Progesterone Metabolism on Dilauroylphosphatidylcholine. Addition of dilauroylphosphatidylcholine to the reconstituted system resulted in a stimulation of P-450-dependent progesterone metabolism by all isozymes except P-450b and P-450e (Figure 2A). The maximum effect was observed between 8 and 32 μM for metabolism by P-450c, P-450g, and P-450h (Figure 2B–D). The 16α-hydroxylase activity associated with P-450e was unaffected by dilauroylphosphatidylcholine, while the catalytic activity exhibited by P-450b was inhibited at all phospholipid concentrations investigated (1.6–80 μM), with an approximately 50% inhibition at 8.0 μM (Figure 2A). As noted, the other isozymes were not inhibited by this phospholipid at concentrations of less than 16 μM; therefore, the inhibition cannot be due to a partitioning of progesterone into the lipid and must be the result of a specific interaction with P-450b. Different P-450b preparations produced the same results (data not shown). Finally, when testosterone was 16α-hydroxylated simultaneously in an experiment with progesterone, testosterone monooxygenase activity was modestly stimulated by dilauroylphosphatidylcholine while progesterone was inhibited (Figure 2A). These results indicate that a unique enzyme–substrate interaction

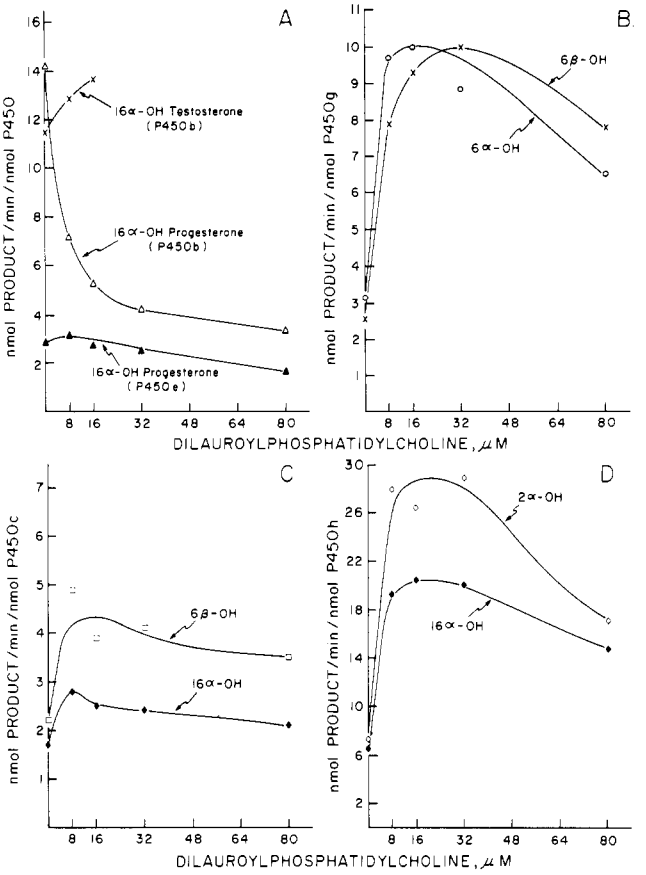

FIGURE 2: Effect of dilauroylphosphatidylcholine on the rate of progesterone metabolism by reconstituted cytochromes P-450: (A) P-450b metabolism of progesterone and testosterone and P-450e metabolism of progesterone; (B) P-450g metabolism of progesterone; (C) P-450c metabolism of progesterone; (D) P-450h metabolism of progesterone. Catalytic conditions are provided under Experimental Procedures. Testosterone was analyzed by the method of Wood et al. (1984).

exists between P-450b, NADPH–cytochrome P-450 reductase, and progesterone that has not been observed with other cytochrome P-450 isozymes, nor has it been observed with P-450b-mediated metabolism of other substrates. It has been postulated that a role of lipid in microsomal reactions is to increase the ability of cytochrome P-450 and NADPH–cytochrome P-450 reductase to form a binary complex necessary for catalytic competence (Strobel et al., 1970; Coon et al., 1976; Miwa & Lu, 1981). It is obvious from this study that this role does not apply to progesterone metabolism by the

Table III: Oxidation of Progesterone Catalyzed by Hepatic Microsomes from Long–Evans Rats[a]

treatment	specific content[b]	total metab[c]	progesterone metabolites														
			2α	2β	6α	6β	7α	11α	11β	12α	14α	15α	15β	16α	16β	17	19	21
immature	0.82	3.57	–	0.21	0.03	1.76	0.50	–	–	–	–	0.04	–	0.61	–	0.14	–	0.25
male		2.93	–	0.17	0.02	1.44	0.41	–	–	–	–	0.03	–	0.50	–	0.11	–	0.21
immature	2.06	3.83	0.12	0.12	0.03	1.99	0.37	–	–	–	–	0.03	–	0.92	–	0.03	–	0.20
Pb male		7.89	0.25	0.25	0.06	4.10	0.76	–	–	–	–	0.06	–	1.90	–	0.06	–	0.41
immature	1.59	2.15	0.04	–	0.03	0.92	0.64	–	–	–	–	0.03	–	0.38	–	0.03	–	0.05
MC male		3.42	0.06	–	0.05	1.46	1.02	–	–	–	–	0.05	–	0.60	–	0.05	–	0.08
adult male	0.83	4.26	0.96	0.10	0.02	1.18	0.11	–	–	–	–	0.05	–	1.63	–	0.02	–	0.17
		3.53	0.80	0.08	0.02	0.98	0.09	–	–	–	–	0.04	–	1.35	–	0.02	–	0.14
adult Pb	1.94	4.23	0.29	0.27	0.01	2.01	0.08	–	–	–	–	0.02	–	1.31	–	–	–	0.22
male		8.21	0.56	0.57	0.02	3.90	0.16	–	–	–	–	0.04	–	2.54	–	–	–	0.43
adult MC	1.35	2.50	0.35	–	0.02	1.04	0.12	–	–	–	–	–	–	0.87	–	0.02	–	0.06
male		3.38	0.47	–	0.03	1.40	0.16	–	–	–	–	–	–	1.17	–	0.03	–	0.08
adult female	0.55	1.25	–	0.05	0.03	0.42	0.37	–	–	–	–	0.04	–	0.20	–	–	–	0.13
		0.69	–	0.03	0.02	0.23	0.20	–	–	–	–	0.02	–	0.11	–	–	–	0.07
adult Dex	1.14	10.59	0.16	1.17	0.07	7.05	0.31	–	–	–	–	0.13	–	0.86	–	0.26	0.15	0.36
female		12.07	0.18	1.33	0.08	8.04	0.35	–	–	–	–	0.15	–	0.98	–	0.30	0.17	0.41

[a] Microsomes containing 0.2 nmol of cytochrome P-450 were incubated with 250 μM progesterone and 1 mM NADPH for 5 min at 37 °C. Dashes indicate that no detectable amount of product was observed (<0.01 nmol min^{-1} $nmol^{-1}$). Pb is phenobarbital-treated, MC is 3-methylcholanthrene-treated, and Dex is dexamethasone-treated rats. Animals were treated with these inducers and microsomes prepared as previously described. Values represent the mean of duplicate determinations. [b] nmol of P-450/mg of protein. [c] First row is in nmol of product min^{-1} (nmol of P-450)$^{-1}$. Second row is in nmol of product min^{-1} (mg of protein)$^{-1}$.

reconstituted system containing P-450b. Another interesting observation is that P-450e, which differs from P-450b in only 13 of 491 amino acids, does not show this phospholipid inhibition.

Determination of Kinetic Constants. To investigate the dilauroylphosphatidylcholine inhibition of progesterone metabolism by P-450b, we evaluated the effect of phospholipid on progesterone binding to P-450b (K_s). To our surprise, no binding spectra, as monitored by $\Delta OD_{360-660nm}$, were observed on addition of progesterone (1–100 μM added in a total of 20 μL of methanol) to P-450b (1 μM) either in the absence or in the presence of dilauroylphosphatidylcholine (32 μM) and/or NADPH–cytochrome P-450 reductase (2.3 μM), indicating that observation of a type I or type II binding spectrum (Schenkman et al., 1981) is not a prerequisite for catalytic activity.

The kinetic constants (K_m^{app} and V_{max}) were determined for progesterone monohydroxylation by P-450b, P-450g, and P-450h under conditions in which less than 15% of the substrate was metabolized. The K_m^{app} associated with 16α-hydroxylation of progesterone by P-450b (Figure 3) was observed to be independent of dilauroylphosphatidylcholine, while V_{max} was decreased 2-fold in the presence of this lipid. The Michaelis constants associated with progesterone metabolism catalyzed by P-450g and P-450h were approximately 50-fold lower than those observed with P-450b. The apparent K_m's for 2α- and 16α-hydroxylation of progesterone by P-450h were 0.66 μM (linear regression analysis, $r^2 = 0.97$) and 0.56 μM ($r^2 = 0.97$), respectively, and those for 6β- and 16α-hydroxylation by P-450g were 0.42 μM ($r^2 = 0.95$) and 0.66 μM ($r^2 = 0.99$), respectively.

From the above data the importance of establishing optimum substrate and phospholipid concentrations for the determination of catalytic activity is apparent. Waxman (1984) reported that the progesterone 16α-hydroxylase activity associated with P-450b was 1.0 nmol min^{-1} (nmol of P-450b)$^{-1}$. That rate is very low in comparison to our value [17 nmol min^{-1} (nmol of P-450b)$^{-1}$] in part because (1) the phospholipid concentration used (7 μg/mL) was inhibitory and (2) the substrate concentration was at K_m^{app} (25 μM). Furthermore, the same substrate concentration was used in the earlier study

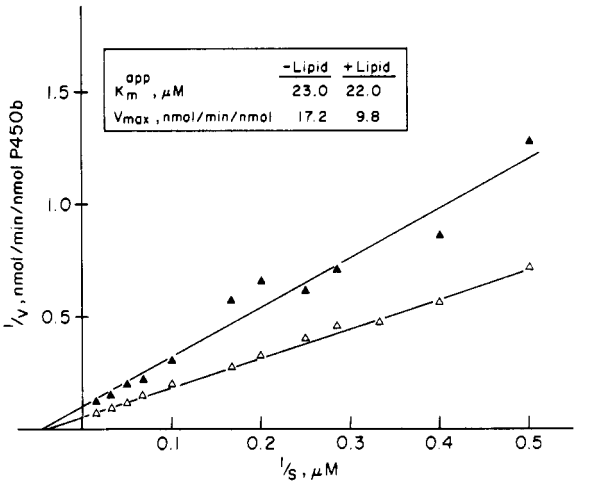

FIGURE 3: Lineweaver–Burk plots of the rate of 16α-hydroxylation of progesterone catalyzed by reconstituted cytochrome P-450b with or without dilauroylphosphatidylcholine. Incubations contained 0.03 μM cytochrome P-450b, 0.4 μM NADPH–cytochrome P-450 reductase, and, when present, 16 μM phospholipid [+lipid (▲); –lipid (△)]. Reactions were for 5 min at 37 °C. Curve fitting was by an unweighted least-squares linear regression analysis, and r^2 values were >0.96.

to determine the catalytic activity of another isozyme (P-450h), even though P-450h has a much lower K_m^{app} (0.6 μM) than P-450b. Thus, comparisons of relative efficiencies of isozymes for specific substrates at nonoptimum conditions can be misleading.

Microsomal Metabolism of Progesterone. The results obtained upon microsomal metabolism of progesterone are shown in Table III. All of the UV-detectable metabolites observed comigrated with authentic monohydroxy standards except for two peaks, one of which was formed exclusively by microsomes from dexamethasone-treated female rats. This metabolite (retention time 43.1 min, HPLC system I) was identified as 6-dehydroprogesterone by comigration with authentic standard as well as by a maximum UV absorption at 284 nm, similar to that previously observed for formation of 6-dehydrotestosterone from testosterone (Nagata et al., 1986). The other peak that comigrated with 15α-OHP on HPLC system I but

Table IV: Antibody Inhibition of Progesterone Metabolism by Hepatic Microsomes[a]

microsomes	antibody	mg of IgG/nmol of P-450	hydroxylated progesterone metabolites (nmol min^{-1} nmol^{-1} or % control)				
			2α + 2β[b]	6β	7α	16α	21
adult control	control		0.920	1.192	0.074	2.021	0.264
	anti-a	5.0	120	103	8	106	101
	anti-g	10.0	79	105	ND	87	96
	anti-h	8.0	12	127	127	38	140
	anti-k[c]	8.0	8	99	142	25	53
adult male Pb	control		0.201 + 0.188	2.878	0.084	1.472	0.300
	anti-b (B50)	0.3	109	108	99	64	137
	anti-h	8.0	8 + 133	114	99	82	95
	anti-k[c]	8.0	2 + 131	108	88	64	20
adult male MC	control		0.143	0.934	0.096	0.638	0.041
	anti-a	5.0	92	101	0	96	83
adult female control	control		0.049	0.440	0.305	0.133	0.154
	anti-a	5.0	ND	86	2	88	83
immature male control	control		0.167	1.953	0.425	0.586	0.254
	anti-a	5.0	102	110	2	102	107
	anti-b (B50)	0.3	98	105	106	97	98
immature male Pb	control		0.249	2.742	0.420	1.165	0.294
	anti-b (B50)	0.3	98	116	112	52	127
	anti-d + C8	5.0 + 0.3	101	112	106	104	109
immature male MC	control		0.057	1.881	0.848	0.627	0.091
	anti-c (C8)	0.3	143	104	143	65	150
	anti-d + C8	5.0 + 0.3	141	109	124	68	132

[a] ND not determined. A total of 0.2 nmol of microsomal P-450 was preincubated with either the appropriate control IgG or anti-P-450 IgG for 10 min at room temperature; 10-min incubations carried out at 37 °C and analytical procedures are as described under Experimental Procedures. Polyclonal or monoclonal (C8 and B50) antibodies have been previously characterized. Pb, phenobarbital; MC, 3-methylcholanthrene. [b] 2α-OHP and 2β-OHP are expressed as the sum of the two metabolites with the exception of microsomes from phenobarbital-treated adult male rats inhibited with anti-h and anti-k. [c] Anti-k recognizes its immunochemically related family of isozymes as described under Experimental Procedures.

not system II was not a hydroxy product as determined by mass spectrometry, and further identification was not attempted.

The age and sex of the rats had a marked effect on the regioselectivity of progesterone oxidation as well as the rate of total metabolism. The primary regioisomers formed by microsomes from immature male rats, 6β-, 7α- and 16α-OHP, were formed at approximately a 3:1:1 ratio. The overall rate of oxidation by the microsomes from mature male rats was not significantly different from the immature animal, but a large change in product regioselectivity occurred such that there was a dramatic increase in 2α-OHP and a lesser increase in 16α-OHP. 7α-OHP formation decreased markedly when metabolism in the adult was compared with that in the immature animal. Microsomes from adult female rats were significantly less efficient (70%) than microsomes from adult male animals in the overall oxidative metabolism of progesterone. Another detectable product was 21-OHP, which was formed at a similar rate per nanomole of P-450 in immature and adult males and adult females [0.13–0.25 nmol min^{-1} (nmol of P-450)$^{-1}$].

Treatment of animals with certain xenobiotics is known to influence the isozymic composition of hepatic cytochromes P-450, and as a result, the regioselectivity of steroid hydroxylation is altered. Phenobarbital administration to both immature and adult males resulted in little change in overall rate of metabolism per nanomole of P-450 but a significant increase (>2-fold) per milligram of protein. Though the regioselectivity of oxidation was similar in untreated and phenobarbital-treated immature animals, the product ratios changed dramatically when phenobarbital was given to adult animals. The major metabolites, 2α-, 6β-, and 16α-OHP, were formed in a ratio of 1:7:5 with phenobarbital-treated adult males as compared to 2:2:3 with untreated animals, and 2β-OHP was observed as a significant metabolite. 21-Hydroxylation was not induced by phenobarbital administration when expressed as nanomoles of product per nanomole of P-450 but was induced 2–3-fold when expressed as nanomoles of product per milligram of protein.

The effects of 3-methylcholanthrene on microsomal metabolism of progesterone in immature and adult male rats were modest. There was a decrease in overall metabolism expressed per nanomole of P-450, but no change per milligram of protein. 7α-Hydroxylation by immature animals was slightly increased by 3-methylcholanthrene induction while 6β- and 16α-hydroxylation decreased as compared to that in untreated animals. The mature males formed 6β-OHP at an equivalent rate following 3-methylcholanthrene treatment compared to untreated adult males, but the rate of formation of 2α-OHP and 16α-OHP was decreased by 50%. 3-Methylcholanthrene treatment also resulted in a 3–5-fold decrease in the rate of 21-hydroxylation in microsomes from immature and adult male rats.

Microsomes from adult female rats treated with dexamethasone oxidized progesterone at a greatly stimulated rate—>8-fold per nanomole of P-450 and >17-fold per milligram of protein compared to untreated females. The rate of formation of all regioisomers formed by untreated females except 7α-OHP was increased by dexamethasone treatment. Since female rats have high NADPH 5α-reductase activity (Yates et al., 1958), it is not known if some of the increases observed after dexamethasone treatment of female rats are due to a repression of this enzyme.

To help define the contribution of an individual isozyme to microsomal metabolism, monospecific inhibitory antibodies raised against the purified cytochromes P-450 were incubated with microsomal preparations (Table IV). Polyclonal monospecific anti-P-450a inhibited >90% of 7α-OHP formation in microsomes from control adult males, 3-methylcholanthrene-treated adult males, control adult females, and control immature males, which is consistent with P-450a being the only known progesterone 7α-hydroxylase. A monoclonal antibody raised against P-450b (B50) inhibited 16α-hydroxylation in microsomes from adult and immature males treated with phenobarbital by 36% and 48%, respectively. No inhibition of progesterone activity by B50 was observed in

microsomes from untreated immature male rats. Monoclonal anti-P-450c (C8) alone or in combination with polyclonal anti-P-450d inhibited 32–35% of the 16α-hydroxylase activity catalyzed by microsomes from immature males treated with 3-methylcholanthrene but did not inhibit 6β-hydroxylase activity, which is also catalyzed by P-450c and P-450d. The observation that an antibody specific for P-450c gave the same inhibition of 16α-hydroxylase as anti-P-450c+d indicates that the inhibited activity is due to P-450c and not P-450d. A mixture of C8 and anti-P-450d was also incubated with microsomes that are essentially devoid of P-450c and P-450d, i.e., microsomes from phenobarbital-treated immature males, to check for nonspecific inhibition of 16α-hydroxylation, but no inhibition was observed. When polyclonal monospecific anti-P-450g was incubated with microsomes from control adult males, no inhibition was observed. Both 16α- and 2α-hydroxylation were inhibited by antibody against P-450h, an enzyme with equivalent 2α- and 16α-hydroxylase activity. 2α-Hydroxylation was inhibited 90% in microsomes from either untreated or phenobarbital-treated rats, while 16α-hydroxylation was inhibited 62% and 18%, respectively. Unfortunately, this laboratory does not have a monospecific antibody that recognizes only P-450k. An antibody was used that is known to react with P-450h, P-450g, P-450f, and P-450i, the immunochemically related proteins, as well as P-450k. This antibody was used to determine the potential contribution of P-450k to microsomal 21-hydroxylation since none of the other isozymes catalyzed this reaction. 21-Hydroxylation of progesterone was inhibited 80% in microsomes from phenobarbital-treated adult males by this antibody and 47% in microsomes from control adult males. 2α- and 16α-hydroxylation were also observed to be inhibited by anti-P-450k as expected since this antibody also inhibits P-450h-mediated metabolism. These data indicate that P-450k or a protein immunochemically related to P-450k is responsible for much of the 21-hydroxylation in microsomes from male rats.

Discussion

Metabolism of steroids by hepatic cytochromes P-450 is of importance to determine the role of hepatic enzymes in steroid hormone homeostasis and as a method of characterizing and identifying structural features of these enzymes. The 11 purified isozymes investigated in this study converted progesterone with overlapping selectivity to 7 regioisomers, while the 8 microsomal preparations converted progesterone to 10 regioisomers. The effects of age, sex, and inducer upon isozymic composition were illuminated in the metabolite profiles derived from the various microsomal preparations as well as the antibody inhibition experiments.

Steroid A ring hydroxylation of progesterone by the reconstituted systems studied was limited to oxidation of the 2α-carbon–hydrogen bond. Cytochromes P-450h and P-450f were the only isozymes observed to have this catalytic specificity, although P-450h was greater than 80-fold more active than P-450f. The microsomal expression and antibody inhibition of progesterone 2α-hydroxylase activity correlated with the reported hepatic content of P-450h (Waxman et al., 1985) and the assertion that this isozyme is the predominant hepatic steroid 2α-hydroxylase (Waxman, 1984). Although none of the reconstituted enzymes catalyzed 2β-oxidation, this activity was detected in hepatic microsomes from immature and adult males that were untreated or phenobarbital treated as well as adult females that were untreated or administered dexamethasone. No measurable 2β-hydroxylase activity was observed in microsomes from immature or adult male rats treated with 3-methylcholanthrene. This activity was markedly inducible by dexamethasone in adult female animals (Table III). The induction of 2β-hydroxylase activity correlates with the level of P-450p induction reported for microsomes from control and dexamethasone-treated female rats (Heuman et al., 1982) and suggests that 2β-hydroxylation may be a steroid hydroxylase activity specific for this isozyme. To date, purified preparations of P-450p have had very low catalytic activity; thus, confirmation of the microsomal activity has not been established (Elshourbagy & Guzelian, 1980; Guengerich et al., 1982; Waxman et al., 1985). It is of note that although the progesterone 2α-hydroxylase activity associated with P-450h has previously been reported (Waxman, 1984; Cheng & Schenkman, 1984), this is the first report to distinguish 2α- from 2β-hydroxylase activity. The data in our study conclusively show that P-450h has no 2β-hydroxylase activity and identify 2β-hydroxylation as a significant reaction in several microsomal preparations.

The progesterone B ring was oxidized by cytochrome P-450 to form three hydroxylated products—6α-OHP, 6β-OHP, and 7α-OHP. Cytochrome P-450a alone catalyzed hydroxylation of progesterone to the 6α and 7α regioisomers at a ratio of 1:14. 7α-Hydroxylation of progesterone was inhibited >90% in all microsomes that were incubated with anti-P-450a, and the microsomal rate of 7α-hydroxylase activity correlated with the specific content of microsomal P-450a determined by immunoquantitation (Thomas et al., 1981). The 6β regioisomer was formed by four of the isozymes investigated—P-450g, P-450h, P-450c, and P-450d. This metabolite has also been reported to be formed by RLM2 (Jansson et al., 1985), an uninducible rat hepatic protein, as well as by a PCN-inducible protein (Einarsson & Gustafsson, 1973). Although 6β-hydroxylation was a major product of progesterone metabolism by all microsomal preparations investigated, monospecific inhibitory antibodies against P-450c, P-450d, P-450g, and P-450h did not inhibit 6β-hydroxylase activity. However, an inhibitory antibody to P-450p inhibited greater than 80% of 6β-hydroxylation of progesterone catalyzed by microsomes from adult male rats (data not shown). These data suggest that P-450p is a major microsomal 6β-hydroxylase and that 6β-hydroxylation catalyzed by P-450g does not contribute significantly in microsomes even though purified P-450g in the reconstituted system exhibits considerable activity.

Only two D-ring regioisomers of progesterone were observed in this study—15α-OHP and 16α-OHP. 15α-OHP was formed at a nominal rate by the sex-specific isozymes P-450g, P-450h, and P-450i as well as most of the microsomal preparations investigated. Hydroxylation of progesterone at the 16α-position was the least specific site of metabolism since 8 of the isozymes exhibited 16α-hydroxylase activity. Only P-450a, P-450i, and P-450j were devoid of this activity, and P-450i and P-450j are essentially inactive toward progesterone. 16α-Hydroxylation of testosterone (Reik et al., 1985) and androstenedione (Waxman et al., 1985) has been extensively investigated and associated with P-450h in adult male control animals, as well as P-450b and P-450e in phenobarbital-induced male animals. The results of antibody inhibition studies indicate that P-450h is a major progesterone 16α-hydroxylase in microsomes from control adult male rats (>60% inhibition by anti-P-450h). However, less antibody inhibition of P-450b-mediated 16α-hydroxylation was observed when progesterone was the substrate compared to testosterone. Testosterone 16α-hydroxylase activity was induced 10-fold by phenobarbital treatment of immature male rats and was completely inhibited (>90%) by monoclonal anti-P-450b (B50)

Table V: Comparison of Steroid Activities Associated with Purified Cytochrome P-450 Isozymes[a]

	progesterone		testosterone		androstenedione	
isozyme	metabolites	ratio	metabolites	ratio	metabolites	ratio
a	7α/6α	14/1	7α/6α	21/1	7α/6α	30/1
b	16α		17α/16β/16α	1.5/1/1.3	16α/16β	1/13
c	16α/6β	1/2	6β		6β	
d	16α/6β	1/4	6β		6β	
e	16α		17α/16β/16α	1.6/1/1.1	16α/16β	1/12
f	16α/2α	2/1	16α		ND	
g	16α/15α/6β	20/1/17	16α/15α/6β	1/3/13	16α/6β	1/7
h	16α/15α/6β/2α	102/1/9/132	16α/6β/2α	40/1/37	16α/6β/2α	50/4/1
i	15α		15α/1α	1/1	ND	
j	–		–		ND	
k	21/16α	1/1	–		ND	

[a] Dashes indicate that no significant metabolites were detected [≤0.1 nmol min^{-1} (nmol of P-450)$^{-1}$]. ND, not determined. Androstenedione and testosterone metabolism by P-450a, P-450b, P-450c, P-450d, and P-450e (Wood et al., 1983); testosterone metabolism by P-450f, P-450g, and P-450h (Ryan et al., 1984a), P-450i (Ryan et al., 1984b), P-450j (Ryan et al., 1985), and P-450k (Waxman & Walsh, 1983); androstenedione metabolism by P-450a and P-450h (Waxman, 1984) and P-450g (McClellan-Green et al., 1987).

(Reik et al., 1985). On the other hand, progesterone activity was induced only 50% and inhibited only 36% by monoclonal anti-P-450b (B50). This difference could be a result of a significant contribution by another isozyme and/or a lack of activity associated with P-450b in 16α-hydroxylation of progesterone. Lipid studies in the reconstituted system indicate that dilauroylphosphatidylcholine inhibits P-450b-catalyzed progesterone metabolism; accordingly, if this inhibition by lipid were to occur in microsomes, the result would be a lack of pronounced induction of 16α-hydroxylase activity even though P-450b is markedly induced by phenobarbital.

The microsomal content of the other major progesterone 16α-hydroxylases, P-450h and P-450g, is minimal in immature animals and is not induced by phenobarbital (Waxman et al., 1985; Bandiera et al., 1986; Maeda et al., 1984). Cytochrome P-450p, an isozyme known to be induced by phenobarbital and present in noninduced animals (Heuman et al., 1982; Waxman et al., 1985), may have 16α-hydroxylase activity for progesterone as suggested by the 4-fold induction of this activity in dexamethasone-treated females. If so, this isozyme may contribute significantly to the observed activity in microsomes from phenobarbital-treated animals.

Cytochromes P-450c and P-450d also have 16α-hydroxylase activity toward progesterone. The microsomal content of these isozymes in noninduced and phenobarbital-induced animals is very low (Thomas et al., 1983), thus eliminating a significant contribution from these isozymes. This conclusion is consistent with the lack of inhibition of 16α-hydroxylase activity by anti-P-450c (C8) and anti-P-450d in microsomes from immature male rats treated with phenobarbital. Interestingly, this antibody mixture or anti-P-450c (C8) alone slightly inhibited 16α-hydroxylation activity in microsomes from 3-methylcholanthrene-treated immature animals (32–35%) although 6β-hydroxylation did not appear to be affected.

This is the first study to have 16β-OHP available as a standard and to conclusively eliminate it as a potential product of progesterone metabolism by several cytochrome P-450 isozymes and microsomes from certain treated animals. This is a point of interest because 16β-hydroxylation of testosterone and androstenedione has been reported to accompany 16α-hydroxylation by P-450b and P-450e (Wood et al., 1983).

The reconstituted enzyme systems also did not catalyze detectable formation of 14α-OHP, 15β-OHP, or 17α-OHP, whereas 17α-OHP was formed minimally by some microsomal preparations. Jansson et al. (1985) have reported that RLM2 preferentially hydroxylates progesterone at the 15-position with 15β-hydroxylation accounting for 34% of product formation and 15α-OHP for 13%. They reported that RLM3, a protein analogous to P-450g, also appears to catalyze 15β-hydroxylation (Cheng & Schenkman, 1984; Jansson et al., 1985). 15β-Hydroxylation of progesterone has also been observed to occur in microsomes from adult male rats and perfused liver (Gustafsson & Lisboa, 1970; Eriksson et al., 1972). The discrepancy between the results reported here and those of previous studies is unknown. But in this study, 15β-OHP, which was synthesized, characterized by mass spectrometry and NMR, and resolved from all other regioisomers, was not detectable as a metabolite.

Of the three methyl groups on the steroid nucleus (C-18, C-19, and C-21), only C-21 was hydroxylated by a purified isozyme (P-450k). C-21 hydroxylation activity was observed in all microsomal preparations, and C-19 only in microsomes from dexamethasone-treated adult female rats. 21-Hydroxylation activity was induced >2-fold per milligram of protein by phenobarbital and dexamethasone and repressed by 3-methylcholanthrene. The rate of 21-hydroxylation of progesterone correlates with the reported induction of P-450k by phenobarbital (Guengerich et al., 1982; Waxman et al., 1985), suggesting that this isozyme could contribute to microsomal 21-hydroxylase activity. Indeed, 21-hydroxylation activity was inhibited 47% in microsomes from adult control males and 80% in microsomes from phenobarbital-treated animals by anti-P-450k, indicating that P-450k or an immunochemically related protein participates in progesterone 21-hydroxylation.

It is obvious from the data reported here that the regioselective hydroxylation of progesterone by the cytochrome P-450 isozymes is strict and well-defined. To evaluate the effects of substrate binding on product regioselectivity, we have compared the data obtained here on progesterone with those previously obtained for cytochrome P-450- dependent metabolism of testosterone and androstenedione (Table V). The molecules are identical except for the substituent at carbon 17—a 17β-acetyl, 17β-hydroxy, and 17-keto substituent for progesterone, testosterone, and androstenedione, respectively. Insight as to the role of D ring–protein interactions upon catalytic conversion can be achieved by investigating changes in the regioselectivity of product formation elicited by the subtle alteration of steroid structure.

The regioselectivity of steroid hydroxylation associated with P-450a was relatively insensitive to D-ring substitutions although the rate of metabolism of the three steriods by this isozyme varied 2–3-fold. Thus, the unique 7α-hydroxylase activity of P-450a must not be determined principally by interactions between the steroid D ring and the protein. Two phenobarbital-inducible proteins, P-450b and P-450e, catalyze

only D-ring hydroxylation of the three steroids. As expected, the regioselectivity of D-ring hydroxylation is greatly affected by the substituent. The greater the molar volume of the 17β substituent the less 16β-hydroxylation observed. Changes in the ratio of 16α to 16β product formed were >200, 1.2, and 0.08 for progesterone, testosterone, and androstenedione, respectively. The regioselective formation of only D-ring regioisomers suggests that the size of the substituent does not affect the general orientation of binding, i.e., D-ring bound specifically in the vicinity of heme, but that the increased size of the 17β-acetyl substituent most likely blocks access to the 16β position either by prevention of rotation in the active site or through steric hindrance by the 17β-acetyl group.

The major 3-methylcholanthrene-inducible isozymes, P-450c and P-450d, formed two products, 6β-OH and 16α-OH, when progesterone was substrate, while only one hydroxylated product (6β) was observed with the other substrates (Table V). Recent studies evaluating the enantiomeric composition of the polycyclic aromatic hydrocarbon metabolites formed by cytochrome P-450c have provided a stereochemical model for the active site of this protein (Jerina et al., 1982). As previously reported (Wood et al., 1983), 6β-hydroxylation of androstenedione and testosterone is consistent with the active site model as is 6β-hydroxylation of progesterone. 16α-Hydroxylation of these steroids is also consistent with the model although the factors that lead to 16α-OHP formation but not 16α-hydroxyandrostenedione or 16α-hydroxytestosterone are unclear. Possibly a group exists in the active site of P-450c that can hydrogen bond to a substrate to elicit another allowed conformation, and while progesterone has the appropriate intramolecular distance to bind in this catalytic orientation, androstenedione and testosterone do not.

Cytochromes P-450g and P-450h can bind the substrates in two or more allowable catalytic conformations. For P-450g, which can bind substrates in two catalytic conformations, one leading to 6β products and one leading to D-ring products, addition of an acetyl group (progesterone) increases the amount of D-ring 16α-hydroxylation as compared to 6β-hydroxylation. The observed dramatic changes in product ratios with no change in the regioisomers formed suggest that two distinctly different conformations exist for this protein, and subtle changes in substrate will vary the equilibrium rates between these conformations. These data are suggestive of a functional group in the active site of P-450g that can hydrogen bond with the two carbonyl groups of progesterone allowing two conformations with the proper orientation for substrate hydroxylation. The hydrogen atoms of the 6- and 16-carbons, which are abstracted in the first step of hydroxylation, and the carbonyl oxygens are separated by a similar spacial distance (5–6 Å), suggesting a hydrogen-bonding group, if present, to be approximately 7–10 Å from the activated oxygen. Thus, hydrogen bonding to the 3-carbonyl would facilitate 6β-hydroxylation and hydrogen bonding to the 20-carbonyl would facilitate D-ring hydroxylation. In addition, 6β-hydroxylation would be predicted by this proposal to be more prevalent than 16α-hydroxylation with compounds that do not have a 20-carbonyl group. Indeed, this is what is observed in P-450g-catalyzed hydroxylation of androstenedione and testosterone.

A similar phenomenon occurs with P-450h where three distinct catalytic conformations are observed, one leading to 2α-hydroxylation, one to 6β-hydroxylation, and one to D-ring hydroxylation (16α). Progesterone and testosterone bind in all three conformations, while androstenedione binds primarily in one catalytic conformation—that leading to 16α-hydroxylation. It is interesting to note that while P-450g- and P-450h-catalyzed hydroxylation of progesterone is less regioselective than P450b-catalyzed hydroxylation, these enzymes have a much greater affinity for the substrate than P-450b as suggested by the approximately 30–40-fold difference in K_m^{app}. Therefore, it may be that these two constitutive isozymes have more specific interactions (i.e., hydrogen bonding) with the steroid substrates than the inducible enzyme whose interactions with the substrate may be mostly hydrophobic in nature. While these data indicated that the active sites of the P-450's have strict boundaries for steroid substrates, more steroids must be investigated before a greater understanding of these catalytic conformations can be deciphered.

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