The cytochrome P-450-dependent monooxygenase system oxidizes a vast number of structurally diverse lipophilic compounds to more polar organic molecules, thereby facilitating their excretion from the body (1). Cytochrome P-450 is the terminal electron acceptor and substrate-binding moiety of this enzyme complex, and the unusually broad substrate specificity of the monooxygenase system is due in large part to the existence of multiple forms of the hemoprotein with different but overlapping substrate specificities (2, 3). The relative amounts of the individual cytochrome P-450 isoforms are markedly dependent on the sex, species, age, and genetic constitution of the animal, as well as on prior exposure to inducers of the hemoproteins (1, 2, 4).

To date, this laboratory has described the purification of five distinct cytochrome P-450 isoforms from hepatic microsomes of immature male Long-Evans rats (3, 5-7). The isoforms, designated cytochromes P-450a, P-450b, P-450c, P-450d and P-450e, have been characterized by their minimum molecular weight in sodium dodecyl sulfate-polyacrylamide gels, spectral, catalytic, and immunological properties, peptide fragments generated by proteolytic enzymes or cyanogen bromide, amino acid compositions, and partial amino acid sequences. Steroid hydroxylase reactions, particularly reactions utilizing testosterone as substrate, have proved useful in characterizing the individual catalytic capacity of the cytochrome P-450 isoforms. However, these studies have utilized a paper chromatographic system for the separation of testosterone metabolites (8), and analyses have been limited to the 6α-, 7α-, and 16α-hydroxytestosterone metabolites. Previous studies utilizing hepatic microsomes had indicated that cytochrome P-450-dependent monooxygenase systems can oxidize testosterone at several additional positions (8-12). Furthermore, it has been shown that the profile of testosterone metabolites generated by microsomal cytochrome P-450 monooxygenases can be markedly influenced by age, sex, species, and environmental factors (12-14). We therefore have undertaken a comprehensive assessment of testosterone oxidation catalyzed by the five highly purified cytochrome P-450 isoforms in order to (i) systematically characterize their catalytic activities and (ii) commence a definitive understanding of the regulation of steroid oxidations by the hepatic hemoproteins. HPLC systems capable of resolving at least 18 known and potential primary oxidative testosterone metabolites, including six pairs of stereoisomers, were utilized for the analyses. Interest in possible stereospecific or stereoselective oxidations was prompted by previous studies which indicated that cytochrome P-450c can metabolize many poly cyclic aromatic hydrocarbons to products with high optical activity (15, 16).

Modifications of the HPLC systems used for the separation of the hydroxysterosterones led to a system capable of analyzing the metabolites of androstenedione. Thus, the regio- and stereoselective oxidations of two C19 steroids by the five cytochrome P-450 isoforms are compared and contrasted in this report. In addition, the steroid metabolite profiles generated by the hepatic microsomes from which the hemoproteins were isolated have been determined.
Steroid Metabolism by Cytochrome P-450 Isozymes

EXPERIMENTAL PROCEDURES

Chemicals—Testosterone and [4-14C]testosterone were obtained from Aldrich and New England Nuclear, respectively. [4-14C]hydroxytestosterone was a gift of G. D. Searle and Co.; 2α-, 2β-, 6α-, 7α-, 7β-, 11α-, 15α-, and 16α-hydroxytestosterone were a gift of the Steroid Reference Collection, MRC, London, United Kingdom; 15β-hydroxytestosterone was a gift of Dr. Theo a. van der Hoeven (Albany Medical College, Albany, NY); and 7β-, 14α-, 18α- and 19α-hydroxytestosterone were a gift of Dr. A. H. Conney (Hospital of the University of Pennsylvania, Philadelphia, PA). [4-14C]Androstenedione and [4-14C]cholestenedione were purchased from Sigma and New England Nuclear, respectively. 6α-, 7α-, 7β-, 11β-, 14α-, 15α-, and 16α-hydroxyandrosterone and 17α-hydroxyandrostenedione were a gift of Dr. A. H. Conney. 16α-Hydroxy- and 18α-hydroxyandrosterone were purchased from Steroids, Inc. (Wilton, NH).

Androstenedione and [4-14C]cholestenedione were purchased from Sigma and New England Nuclear, respectively. 6α-, 7α-, 7β-, 11β-, 14α-, 15α-, and 16α-hydroxyandrosterone were provided by the Steroid Reference Collection; and 7β- and 9α-hydroxyandrosterone and 17α-hydroxyandrostenedione were a gift of Dr. A. H. Conney. 16α-Hydroxyandrosterone was purchased from Sigma.

Dilauroylphosphatidylcholine was obtained from Serdary Research Laboratories, Ontario, Canada. Aroclor 1254 (lot KC-12-638), phenobarbital, and 3-methylcholanthrene were obtained from Monsanto Co. (St. Louis, MO), Merck, and Sigma, respectively. Other biochemicals were obtained from Sigma, and all organic solvents were obtained from Burdick and Jackson Laboratories Inc., Muskegon, MI.

Enzyme Preparations—Immature male Long-Evans rats (50-60 g; Bio-Serv, Inc., Frenchtown, NJ) were treated with 100 mg/kg of phenobarbital (75 mg/kg/day in water) for 4 days and killed 24 h after the last dose. Aroclor 1254 was administered as a single intraperitoneal dose (500 mg/kg in corn oil) 4 days before the animals were killed. Hepatic microsomes were prepared as previously described (17) and stored at -90 °C prior to use. Cytochrome P-450 was determined by the method of Omura and Sato (18). Protein was determined by the method of Lowry et al. (19). Cytochrome P-450a, P-450b, P-450c, and P-450e were purified from hepatic microsomes of Aroclor 1254-treated rats, as described (5, 7), while cytochrome P-450d was purified from hepatic microsomes of phenobarbital-treated rats (6). The isozymes had specific contents of cytochrome P-450 (nanomoles/mg of protein) of 12-16 when protein was measured by the method of Lowry et al. (19) and 16-21 when protein was determined by amino acid compositions (20). One purified cytochrome P-450 isozyme gave a single protein-stainable band on sodium dodecyl sulfate-polyacrylamide gels. NADPH-cytochrome c reductase was purified from hepatic microsomes of phenobarbital-treated rats to a specific activity of 35,000-40,000 units/mg of protein by a modification (5) of the methods of Yashuochi and Masters (21) and Dignam and Strobe1 (22). Unit of reductase catalyzes the reduction of 1 nmol of cytochrome c/min at 22 °C in 0.3 M potassium phosphate buffer (pH 7.7) containing 1 mM EDTA and 0.1 mM NADPH.

Incubation and Extraction Conditions—Incubations with hepatic microsomes and 0.25 to 1.0 nmol of cytochrome P-450, 1.0 mM of NADPH, 5 μmol of magnesium chloride, 50 μmol of sucrose, and 50 μmol of dilauroylphosphatidylcholine were performed in a 1 ml of Tris-HCl buffer (pH 7.4) at 37 °C for 5 min following the addition of substrate. Incubations with the purified cytochrome P-450 isozymes contained from 0.25 to 0.10 nmol of hemoprotein, 1200 units of NADPH-cytochrome c reductase, 10 μg of dilauroylphosphatidylcholine, 1 μmol of NADP, 3 μmol of magnesium chloride, 50 μmol of potassium phosphate buffer (pH 7.4), 125 nmol of testosterone or androstenedione (added in 20 μl of methanol)/1.0-ml final volume, and were agitated at 37 °C for 5 or 10 min. When used, the final specific activity of 4-14C-testosterone was 8.0 μCi/μmol. In experiments designed to examine further oxidation of monoxygenase substrates, the substrate concentration was 66 nmol/1-ml final volume. Catalytic activities of all enzyme preparations were determined under conditions in which metabolism was proportional to cytochrome P-450 concentration and time of incubation. Preliminary experiments indicated that the above stated amounts of NADPH-cytochrome c reductase and dilauroylphosphatidylcholine were optimal for the purified hemoproteins, and the NADPH concentration was saturating under all assay conditions. All reactions were terminated by vortexing the incubation mixtures for 30 s after the addition of 6 ml of methylene chloride. Following centrifugation at 3000 g for 1 min at 800 × g for 10 min at 800 μl of the organic phase were transferred to a culture tube (15 × 100 mm) and evaporated under a stream of nitrogen. The residue was dissolved in 0.05-0.2 ml of methanol and analyzed by HPLC.

High Pressure Liquid Chromatography—All analyses were performed with a Waters Model 2004 liquid chromatograph equipped with a second Waters Model 6000A pump and a Waters Model 660 solvent programmer. Testosterone metabolites were analyzed by modifications of the procedure described by van der Hoeven (9), using a 5-μm octyldecalysine reverse phase column (150 × 4.6 mm inner diameter) (Supelco, Inc., Bellefonte, PA) preceded by a guard column of 30-38-μm octyldecalysine (Whatman) and a 2-μm column inlet filter (Rheo-Cell, Radnor, PA). The eluent was monitored by a Waters 486 detector set at a flow rate of 1.4 ml/min. A second chromatography system was utilized to enhance resolution of 6α- and 10α-hydroxytestosterone and consisted of a 5-μm octyldecalysine reverse phase column (150 × 4.6 mm inner diameter) (DuPont Zorbax) preceded by the above described guard column and filter. The column was eluted isocratically with a mobile phase of 20% tetrahydrydron in water for an additional 5 min. Androstenedione metabolites were analyzed with a chromatography system utilizing the Dupont column and a concave gradient (No. 8, Waters 660 solvent programmer) of 15-36% tetrahydrydron in water for 20 min at a flow rate of 1.6 ml/min. All chromatographic separations were performed at ambient room temperature (22-24 °C), and column effluents were monitored at 254 nm using a Waters Model 440 data collection system equipped with an UV-visible absorbance detector. Metabolites were generally quantitated by comparison of their peak heights with those of authentic standards. When 4-14C-labeled steroids were used as substrates, 0.2-0.3 ml fractions were collected and radioactive metabolites were quantitated by scintillation spectroscopy.

RESULTS

Chromatographic Conditions—The HPLC profile of testosterone and androstenedione, and known potential oxidative metabolites of testosterone utilizing a reverse phase C18 column (Supelco) and a mobile phase consisting of a methanol:water:acetonitrile gradient is illustrated in Fig. 1A. Base-line or near base-line resolution of 13 compounds was achieved while resolution of four pairs of compounds was incomplete. Preliminary experiments with hepatic microsomes as well as the purified isozymes indicated that the incomplete resolution of 1α- and 1β-hydroxytestosterone and 2α- and 11β-hydroxytestosterone was of no consequence since analyses using a modified gradient which resolved these peaks did not provide any evidence for the metabolic formation of one or both constituents of each pair. This chromatographic system, and extensive modifications of it, failed to adequately separate the known and potential metabolites of androstenedione, despite the similarity in structure of the two C19 steroids. Utilization of a high carbon load C18 reverse phase column (DuPont) and a mobile phase consisting of a tetrahydrydron:water gradient resulted in the resolution of androstenedione and 10 of 11 monohydroxy derivatives (Fig. 1B). Fortuitously, this system also served as a basis for the enhanced resolution of 6β-, 7α-, 11β-, 15α-, and 15β-hydroxytestosterone (Fig. 1C).

Oxidation of Testosterone Catalyzed by Purified Cytochrome P-450 Isozymes—Fig. 2 illustrates the composite chromatographic profiles of testosterone metabolites formed by the five individual cytochrome P-450 isozymes under conditions in which metabolite formation was proportional to incubation time and amount of cytochrome P-450; NADPH-cytochrome c reductase was saturating, and phosphatidylcholine concentration was optimal. Turnover numbers, expressed throughout this report as nanomoles of product formed per min/nmol of hemoprotein, were calculated from these profiles and those of additional incubations and are summarized in Table I. Quantitation of metabolites by ultraviolet absorbance at 254 nm and scintillation spectroscopy gave essentially identical values.

The marked regio- and in some cases stereoselectivity of the isozymes is dramatic. Cytochrome P-450a catalyzed the oxidation of testosterone to one major (≥95%) product, 7α-hydroxytestosterone, at a turnover rate of 21. Cytochrome P-450d had a turnover rate of 15.
Steroid Metabolism by Cytochrome P-450 Isozymes

FIG. 1. HPLC profiles of testosterone, androstenedione, and available reference compounds. A, profile of testosterone and 18 known or potential testosterone metabolites eluted from a reverse phase C18 column (Supelco) using a methanol:water:acetonitrile mobile phase. B, profile of androstenedione and 11 known or potential androstenedione metabolites eluted from the DuPont column using a tetrahydrofuran:water mobile phase. C, profile of five testosterone metabolites eluted from a reverse phase C8 column (DuPont) using a tetrahydrofuran:water mobile phase. The arrow at 11 min indicates the time of change in the mobile phase. Mixtures contained approximately 0.3 nmol of each compound, and absorbance at 254 nm was monitored at a sensitivity of 0.02 absorbance units full scale. Details are given under “Experimental Procedures.” A and T are abbreviations for androstenedione and testosterone, respectively, and with the exception of 16α=0 (which represents 16-ketotestosterone), the remaining abbreviations represent monohydroxy derivatives of the parent steroid.

FIG. 2. Composite HPLC profile of testosterone metabolites formed by five cytochrome P-450 isozymes. A Supelco column and the methanol:water:acetonitrile mobile phase described under “Experimental Procedures” were used for the analyses of extracts of 10-min incubations containing 0.1 nmol of each hemoprotein. 16β-OHA, 16β-hydroxyandrostenedione. See the legend to Fig. 1 for definitions of other metabolite abbreviations.

TABLE I

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Oxidation of testosterone and androstenedione catalyzed by five highly purified cytochrome P-450 isozymes

Incubation and analytical conditions were as described under “Experimental Procedures.” Values represent the mean turnover numbers obtained with two or, in some cases, three different amounts of cytochrome P-450 (from 0.025 to 0.10 nmol of hemoprotein). 450b catalyzed the oxidation of testosterone at positions 16 and 17, exclusively. Oxidation at the 16-position occurred with little, if any, stereoselectivity since turnover numbers for 16α- and 16β-hydroxylation were 9.1 and 7.2, respectively. Androstenedione formation, resulting from the oxidation of the 17β-hydroxyl group of testosterone to the 17-keto group, was totally dependent on the presence of hemoprotein and NADPH-cytochrome c reductase, and substitution of NADP for NADPH resulted in a complete loss of product formation. Identification of the metabolite eluting at 11 min as 16β-hydroxyandrostenedione by nuclear magnetic resonance spectroscopy, coupled with the observation that formation of this metabolite exhibited a lag with respect to time of incubation, indicated that androstenedione was being further metabolized by cytochrome P-450b. Summation of the turnover numbers calculated for formation of 16β-hydroxyandrostenedione and androstenedione to give a true value for the total rate of androstenedione formation revealed that the three primary oxidation products resulting from the oxidation of testosterone...
one by cytochrome P-450b are formed in approximately equivalent amounts (Table I). Cytochrome P-450e, which is immunochemically identical with cytochrome P-450b (7) and differs from this isozyme by only 13 of 491 amino acids (25), catalyzed the oxidation of testosterone to the same metabolites as cytochrome P-450b, the major hemoprotein induced by phenobarbital (4, 24), but at approximately one-tenth the rate. Oxidation of testosterone catalyzed by either cytochrome P-450c or P-450d resulted in a single product, 6β-hydroxytestosterone. Because these reactions were thus both regio- and stereospecific, catalysis was relatively inefficient since the turnover numbers were 1.9 and 0.7, respectively. The low catalytic activity of cytochrome P-450d was due to the isosafrole-metabolite complex of the hemoprotein since displacement of this metabolite followed by dialysis of the hemoprotein (6) did not alter its catalytic activity toward testosterone.

**Oxidation of Androstenedione Catalyzed by Purified Cytochrome P-450 Isozymes**—The composite chromatographic profiles of androstenedione metabolites formed under optimal experimental conditions by the five cytochrome P-450 isozymes are illustrated in Fig. 3. To facilitate comparisons between the two C19 steroids, the turnover numbers for the androstenedione metabolites are also summarized in Table I. Androstenedione, like testosterone, was oxidized by cytochrome P-450c, whereas androstenedione catalyzed by cytochrome P-450a regio- and stereospecifically to its 7α-hydroxy metabolite, and the rate of 7α-hydroxyandrostenedione formation was 60% of the rate observed for the 7α-hydroxylation of testosterone. Trace quantities of a metabolite co-chromatographic with testosterone were also detected; turnover numbers of 15 were calculated from the rates of disappearance of 6α- and 7α-hydroxytestosterone. Although the metabolites were not identified, turnover numbers of 15 were calculated from the rates of hydroxylation of the two stereoisomers in the present study. Cytochrome P-450e catalyzed the oxidation of 6β-hydroxytestosterone to 16α-hydroxytestosterone with relatively high efficiency. Although the metabolites were not identified, turnover numbers of 15 and 13 were calculated from the rates of disappearance of 6β- and 16α-hydroxytestosterone, respectively. 16α-Hydroxytestosterone was the only one of the four metabolites that catalyzed its formation. Utilizing the Supelco column and the methanol:acetonitrile:water mobile phase (Fig. 1A), this metabolite had a retention time identical with that of 16α-hydroxytestosterone. Trace quantities of a metabolite co-chromatographic with 16α-hydroxytestosterone were identical. Taken together, these data indicate that the sequential oxidation of testosterone is dependent on more than one cytochrome P-450 isozyme and that the hemoprotein catalyzing the formation of the primary oxidative metabolite is not necessarily capable of catalyzing subsequent oxidations.

![Fig. 3. Composite HPLC profile of androstenedione metabolites formed by five cytochrome P-450 isozymes.](image)

As expected from the metabolic profile of testosterone, the 16-position of androstenedione was a favored site of attack by cytochrome P-450b. However, a dramatic difference in the stereoselectivity and rates of hydroxylation of the two steroids is apparent from a comparison of Figs. 2 and 3 and the turnover numbers in Table I. Whereas the molar ratio of 16α- to 16β-hydroxytestosterone formation was 1.3:1, the molar ratio of 16α- to 16β-hydroxyandrostenedione formation was in excess of 1:10. 16α-Hydroxyandrostenedione was formed at half the rate of formation of 16β-hydroxytestosterone, but 16β-hydroxylation of androstenedione proceeded at five times the rate of 16α-hydroxylation of testosterone, resulting in a total turnover of 62 for androstenedione, compared to 27 for testosterone. This high rate of oxidation of androstenedione to 16β-hydroxyandrostenedione catalyzed by cytochrome P-450b is consistent with the formation of 16β-hydroxyandrostenedione when testosterone oxidation is catalyzed by cytochrome P-450b (Fig. 2), according to the pathway: testosterone → androstenedione → 16β-hydroxyandrostenedione. Oxidation of androstenedione catalyzed by cytochrome P-450e produced the same metabolites in essentially the same product ratios as were observed with cytochrome P-450b, but the rate of oxidation with cytochrome P-450e was approximately one-tenth the rate observed with cytochrome P-450b.

**Further Oxidation of Monohydroxylated Testosterone Metabolites Catalyzed by Purified Cytochrome P-450 Isozymes**—Previous studies utilizing rat hepatic microsomes have shown that in the absence of or at low concentrations of testosterone, 6β-, 7α-, and 16α-hydroxytestosterone are all efficiently metabolized to more polar metabolites (25). The capacity of the five purified cytochrome P-450 isozymes to catalyze these oxidations was therefore evaluated as was the further oxidation of 16β-hydroxytestosterone. Interestingly, neither testosterone was metabolized further by any of the cytochrome P-450 isozymes. Only cytochrome P-450a catalyzed the oxidation of 6β-hydroxytestosterone, a metabolite formed exclusively by cytochromes P-450c and P-450d in the present study. Cytochrome P-450a also oxidized 16β-hydroxytestosterone with relatively high efficiency. Although the metabolites were not identified, turnover numbers of 15 and 13 were calculated from the rates of disappearance of 6α- and 16β-hydroxytestosterone, respectively. 16β-Hydroxytestosterone was the only one of the four metabolites that further metabolized by the same isozyme, cytochrome P-450b, that catalyzed its formation. Utilizing the Supelco column and the methanol:acetonitrile:water mobile phase (Fig. 1A), this metabolite had a retention time identical with that of 16β-hydroxytestosterone and 16β-hydroxyandrostenedione. Since both of these compounds were plausible metabolites of 16β-hydroxytestosterone, the analyses were performed with the DuPont column and tetrahydrofuran:water mobile phase under conditions which permitted the resolution of these two monohydroxylated steroids (Fig. 4). Curve A in Fig. 4 illustrates the elution profile of 16β-hydroxyandrostenedione, 16α-hydroxytestosterone, 16β-hydroxytestosterone (poorly resolved), and androstenedione from a 10-min incubation of testosterone with cytochrome P-450b. The single metabolite peak, observed after the metabolism of 16β-hydroxytestosterone by cytochrome P-450b, eluted prior to 16β-hydroxyandrostenedione and was co-chromatographic with a reference sample of 16α-hydroxytestosterone (Fig. 4, Curve B). The nuclear magnetic resonance spectra of this metabolite and authentic 16α-hydroxytestosterone were identical. Taken together, these data indicate that the sequential oxidation of testosterone is dependent on more than one cytochrome P-450 isozyme and that the hemoprotein catalyzing the formation of the primary oxidative metabolite is not necessarily capable of catalyzing subsequent oxidations.

C19 Steroid Oxidation Catalyzed by Hepatic Microsomes Isolated from Untreated and Inducer-treated Rats—In order to place the data obtained with the purified cytochrome P-450 isozymes in perspective, the oxidation of testosterone and androstenedione by hepatic microsomes from untreated and...
Steroid Metabolism by Cytochrome P-450 Isozymes

Fig. 4. HPLC profile of testosterone metabolites obtained on a DuPont CIS column eluted with a concave gradient (No. 8) of 15–36% tetrahydrofuran in water for 20 min at a flow rate of 1.5 ml/min. Curve A, metabolites formed by the incubation of 0.1 nmol of cytochrome P-450b with 125 nmol of testosterone for 10 min at 37°C. Curve B, profile of metabolites formed by the incubation of 0.1 nmol of cytochrome P-450b with 96 nmol of 168-hydroxytestosterone (168-OHT) for 10 min at 37°C. Retention times of authentic reference compounds are as indicated. Note that this chromatographic system exhibits a poor recovery for small quantities of 16α- and 16β-hydroxytestosterone. 168-OHT, 16β-hydroxyandrostenedione.

Fig. 5. Composite chromatographic profiles of testosterone metabolites formed by four microsomal preparations. Turnover numbers and the -fold induction for the various metabolites are summarized in Table II. While turnover numbers are expressed per nmol of cytochrome P-450, the specific content (nanomoles of cytochrome/mg of protein) of each microsomal preparation is included in Table II so that turnover numbers/mg of microsomal protein may be readily calculated. Testosterone was oxidized by microsomes from untreated animals at an overall rate of 8.7 nmol/min/nmol of total cytochrome P-450, and 12 of 13 metabolites peaks were identified by co-chromatography. 2α-, 6α-, and 7α-hydroxytestosterone and androstenedione represented 4, 68, 12, and 8%, respectively, of total identified metabolites, while 2% or less of total metabolites was accounted for by 1β, 2α-, 6α-, 15α-, 15β-, 16α-, 16β-, or 18-hydroxytestosterone. Hepatic microsomes from phenobarbital-treated rats exhibited a greatly enhanced ability to oxidize testosterone at the 16α- (7.7-fold), 16β- (22-fold), and 17- (3.6-fold) positions. Since oxidation at the 2β- and 18-positions was increased only 20% and formation of the remaining six metabolites was decreased from 15 to 40%, total testosterone metabolism/nmol of cytochrome P-450 was increased by only 30% after phenobarbital treatment. The metabolic profile of testosterone metabolites from incubations with microsomes from 3-methylcholanthrene- and Aroclor 1254-treated rats showed a modest increase in 7α-hydroxytestosterone formation (1.3-fold), but oxidation at nine other positions was decreased from 50 to over 90% and overall metabolism, per nmol of cytochrome P-450, was decreased to half of control values. The 64% decrease in the formation of 6α-hydroxytestosterone, which accounted for two-thirds of the total testosterone oxidation in control microsomes, was particularly important in the decrease in total testosterone oxidation/nmol of hemoprotein observed with microsomes from 3-methylcholanthrene-treated animals. Treatment of rats with the chlorinated biphenyl mixture, Aroclor 1254, resulted in an enhanced rate of testosterone metabolism to 16α- and 16β-hydroxytestosterone (2.3- and 5.0-fold, respectively), a relatively minor decrease in androstenedione formation (11%), and 50–90% decreases in the other metabolites. The net result of these alterations was a 60% decrease in the overall rate of testosterone oxidative metabolism, per nmol of hemoprotein, compared to control microsomes. Despite the marked decrease in formation of 6α-hydroxytestosterone in incubations with microsomes from 3-methylcholanthrene- and Aroclor 1254-treated rats and the marked induction of 16α- and 16β-hydroxytestosterone formation in microsomes from phenobarbital-treated rats, 6α-hydroxytestosterone was the most predominant testosterone metabolite formed by all four microsomal preparations. Under all assay conditions, oxidation at the 2-, 6-, 7-, and 15-positions was associated with a relatively high degree of stereoselectivity (3:1 or greater, major:minor isomer), while...
Steroid Metabolism by Cytochrome P-450 Isozymes

**TABLE II**

Oxidation of testosterone catalyzed by hepatic microsomes from immature male Long-Evans rats

Animals were treated with the inducers listed below as described under "Experimental Procedures," which also describes conditions for sample incubation and analysis. Values represent the mean turnover numbers obtained with two or, in some cases, three different amounts of microsomal cytochrome P-450. Turnover numbers for 6β- and 7α-hydroxylation were calculated by averaging the values obtained using both of the chromatographic systems described for testosterone analysis, while 16α-hydroxylation was determined with the DuPont column and the tetrahydroyfuran:water mobile phase.

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<td>nmol/mg protein</td>
<td>12β 2α 2β 6α 6β 7α 15α 15β 16α 16β 18 A Total</td>
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<td>Untreated</td>
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<td>0.07 0.04 0.29 0.06 6.09 1.04 0.04 0.12 0.19 0.07 0.09 0.65 8.72</td>
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<td>anthrene</td>
<td>(0.14)</td>
<td>(&lt;0.25) (0.39) (1.13) (0.36) (1.27) (0.25) (0.29) (0.39) (0.51) (0.13) (0.28) (0.46)</td>
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<td>Aroclor 1254</td>
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<td>(0.14)</td>
<td>(&lt;0.25) (0.47) (0.02) (0.24) (0.32) (0.25) (0.32) (2.3) (5.0) (0.22) (0.89) (0.41)</td>
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*With the exception of A (androstenedione), the abbreviations denote the hydroxylated testosterone metabolite formed, e.g., 12β-hydroxytestosterone.

**TABLE III**

Oxidation of androstenedione catalyzed by hepatic microsomes from immature male Long-Evans rats

Animals were treated with the inducers listed below as described under "Experimental Procedures," which also describes conditions for sample incubation and analysis. Values represent the mean turnover numbers obtained with two or, in some cases, three different amounts of microsomal cytochrome P-450. All incubations were done at the same time as the analogous testosterone incubations (Table II).

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<tr>
<td></td>
<td>nmol/mg protein</td>
<td>12β 2α 2β 6α 6β 7α 15α 15β 16α 16β Total</td>
</tr>
<tr>
<td>Untreated</td>
<td>0.66</td>
<td>0.04 3.82 1.20 0.70 0.91 6.67</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>2.39</td>
<td>(0.25) 0.01 2.37 0.49 1.31 16.67 20.86</td>
</tr>
<tr>
<td>3-Methylchol-</td>
<td>1.48</td>
<td>0.01 1.61 1.49 0.15 0.48 3.74</td>
</tr>
<tr>
<td>anthrene</td>
<td>(0.25)</td>
<td>(0.25) (0.42) (1.24) (0.21) (0.63) (0.56)</td>
</tr>
<tr>
<td>Aroclor 1254</td>
<td>3.38</td>
<td>0.01 0.88 0.47 0.50 6.83 8.77</td>
</tr>
<tr>
<td>(0.25)</td>
<td>(0.25) (0.29) (0.28) (0.71) (7.62) (1.31)</td>
<td></td>
</tr>
</tbody>
</table>

*Abbreviations denote the hydroxylated androstenedione metabolite formed, e.g., 6α-hydroxyandrostenedione.

**See Footnote b in Table II.**

Comparisons of androstenedione and testosterone metabolism by control microsomes indicated that the 6β- and 7α-positions were the first and second most predominant sites of oxidation, respectively, for both steroids and that total androstenedione metabolism proceeded at three-fourths the rates of testosterone metabolism. The most notable differences in metabolism of the two C19 steroids were (i) the apparently fewer androstenedione metabolites, (ii) the 3-10-fold higher rate of hydroxylation of androstenedione at the 16-position, and (iii) the change in molar ratio of the 16α- and 16β-hydroxy metabolites from almost 3:1 for testosterone to 0.81 for androstenedione. Phenobarbital treatment induced the 16β-hydroxylation of androstenedione 18-fold, which was comparable to the 22-fold induction in 16β-hydroxylation of testosterone. However, since 16β-hydroxyandrostenedione was a much more prominent metabolite of control microsomes, the net result was that 16β-hydroxyandrostenedione was a significantly less stereoselectivity in hydroxylation at the 16-position was observed, particularly after treatment of rats with enzyme inducers.

Fig. 6 and Table III summarize the results of analogous experiments in which androstenedione was incubated with the same four microsomal preparations used to assess testosterone metabolism. Evidence for a total of seven androstenedione metabolites was obtained, five of which were co-chromatographic with authentic reference metabolites. One as yet unidentified metabolite appeared to be produced primarily by control microsomes and was eluted with a relatively short retention time (8.1 min), while the other unknown metabolite had a relatively long retention time (22 min). Total oxidative androstenedione metabolism in control microsomes proceeded at a rate of 67 nmol/min/nmol of cytochrome P-450, and 6β-, 7α-, 16α-, and 16β-hydroxyandrostenedione accounted for 58, 18, 10, and 13%, respectively, of total identified metabolites.
Steroid Metabolism by Cytochrome P-450 Isozymes

Fig. 7. Summary of the oxidative metabolism of testosterone and androstenedione catalyzed by five cytochrome P-450 isozymes isolated from the endoplasmic reticulum of immature male Long-Evans rats. Lower case letters refer to the individual isozymes, and the adjacent numbers represent the turnover numbers expressed as nanomoles of product formed per min/mmol of hemoprotein. 7α- and 16α-hydroxytestosterone were not metabolized by any of the five isozymes. Turnover numbers for the metabolism of 6β- and 16α-hydroxytestosterone by cytochrome P-450a were determined by substrate disappearance since the identity of the metabolites was not determined. OHT, hydroxytestosterone; OHA, hydroxyandrostenedione; 16β-OT, 16-ketotestosterone.

drostenedione accounted for 80% of total androstenedione metabolism, which was induced 3-fold over control values. The predominence of the 16β-hydroxyandrostenedione metabolite in incubations of androstenedione with microsomes from phenobarbital-treated rats has been reported previously by Nakamura and Ueda (26). Similar to results with testosterone, 3-methylcholanthrene treatment was associated with a modest increase in the 7α-hydroxylation of androstenedione and a 40–60% decrease in 6β-hydroxylation and total androstenedione metabolism/nmol of total cytochrome P-450. Also in parallel with results obtained with testosterone, Aroclor 1254 treatment was associated with a marked decrease in the 6β- and 7α-hydroxylation of androstenedione. However, as a result of the significant (7.6-fold) induction in the rate of 16β-hydroxyandrostenedione formation, overall oxidation was increased 30% relative to values obtained with control microsomes.

DISCUSSION

The studies described in this report and summarized schematically in Fig. 7 indicate that cytochrome P-450 isozymes isolated from the endoplasmic reticulum of immature male rats differ widely in both the rate and position at which they catalyze the oxidation of the two C19 steroids. Rates of overall metabolism of testosterone and androstenedione varied by 40- and 200-fold, respectively, among the five isozymes. Differences in turnover number did not result from the general catalytic incompetence of one or more of the isozymes since all five hemoproteins have previously been shown to effectively catalyze the metabolism of one or more other substrates (3). The marked positional specificity and stereoselectivity of the isozymes is evidenced by the findings that cytochrome P-450a catalyzed almost exclusively the 7α-hydroxylation of both steroids, while cytochromes P-450c and P-450d were associated exclusively with 6β-hydroxylation. Cytochromes P-450b and P-450e exhibited the least selectivity, although the sites of oxidation were limited to the 16-position of androstenedione and the 16- and 17-positions of testosterone.

While there is precedent for metabolic attack of substrates at more than one position by purified cytochrome P-450 isozymes (cf. Ref. 27), the possibility exists that cytochromes P-450b and P-450e may attack testosterone exclusively at the 16-position. If 16-hydroxylation were to occur, intramolecular transfer of the 17-hydroxyl hydrogen and loss of water could yield androstenedione and the appearance of direct oxidation at the 17-position. Alternatively, the 17-position may be oxidized directly, as is known to occur in the oxidation of the 3,4-dihydrodiol of benz[a]anthracene to 3-keto-4-hydroxybenz[a]anthracene, catalyzed by cytochrome P-450c (16).

Another interesting feature of C19 steroid oxidation catalyzed by cytochromes P-450b and P-450e is that the functional group at the 17-position dramatically influences both the rate and stereoselectivity of 16-hydroxylation. Conversion of the 17β-hydroxy group of testosterone to the 17-keto group of androstenedione is associated with an approximate 50% decrease in the rate of 16α-hydroxylation and an 800–1100% increase in 16β-hydroxylation. Thus, while the 16-hydroxyl group of testosterone proceeds with little or no stereoselectivity, the 16-hydroxylation of androstenedione is highly stereoselective, in favor of β-hydroxylation. Incubations of the 16α- and 16β-hydroxysteroids in the absence of a monooxygenase system indicated that no detectable epimerization occurs at the 16- or 17-positions, and therefore the product ratios appear to be a true reflection of the stereoselectivity of the cytochrome P-450-dependent oxidation reactions.

The complexity of steroid oxidation is clearly illustrated by the studies examining the further metabolism of monohydroxylated testosterone metabolites. Three different results were observed depending on the metabolite chosen as the substrate. Whereas the formation of 6β-hydroxytestosterone was catalyzed by cytochromes P-450c and P-450d, further metabolism of this product was catalyzed by a different isozyme, cytochrome P-450a. In contrast, cytochrome P-450b catalyzed both the formation and further metabolism of 16β-hydroxytestosterone. None of the five isozymes, however, were capable of metabolizing either 7α- or 16α-hydroxytestosterone. In contrast, results have been obtained with metabolites of polycyclic hydrocarbons where the same cytochrome P-450 isoform (P-450c) efficiently catalyzes the formation and further metabolism of the primary metabolites (15, 16, 27).

The steroid metabolism experiments with hepatic microsomes from untreated and inducer-treated rats were undertaken in order to better understand the contribution of the five cytochrome P-450 isozymes to overall oxidative metabolism of the two C19 steroids. Comparison of the data obtained with the purified isozymes and the microsomal preparations, coupled with previously published results on the immunonquantitation of the individual isozymes in hepatic microsomes (4, 24) lead to several observations. First, comparisons of the data in Tables I and II indicate testosterone is oxidized at a minimum of seven positions in microsomes from untreated rats by cytochrome P-450 isoforms other than the five characterized in this study. Second, the marked 18-22-fold induc-
tion (per nmol of total cytochrome P-450) of microsomal 16β-hydroxylation of both androstenedione (Table III) and testosterone (Table II) after phenobarbital treatment parallels the 28-fold induction of cytochrome P-450b (as a per cent of total cytochrome P-450) in these microsomes (4), and is consistent with the high rate of 16β-hydroxylation catalyzed by cytochrome P-450b (Table I). Furthermore, the 8-fold higher rate of 16β-hydroxylation of androstenedione versus testosterone catalyzed by cytochrome P-450b is comparable to the 11-13 fold higher rate of 16β-hydroxylation of androstenedione versus testosterone catalyzed by microsomal preparations. These findings suggested that 16β-hydroxylation of androstenedione or testosterone might represent a relatively good estimate of cytochrome P-450b content, or, more precisely, cytochromes P-450b and P-450e content since this latter isozyme also catalyzes the oxidation of the steroids in the 16β-position, although at markedly lower rates (Table I). Consistent with this possibility, we have found that incubation of antibody to cytochromes P-450b (and P-450e) at a concentration of 5 mg of IgG/nmol of total cytochrome P-450 inhibits 16β-hydroxylation of testosterone and androstenedione by 85–95% in microsomes from phenobarbital- and Aroclor 1254-treated rats. Similar inhibition by anti-P-450b was also observed for 16α-hydroxylation of testosterone and androstenedione by 85–95% in microsomes from phenobarbital- and Aroclor 1254-treated rats. Similar inhibition by anti-P-450b was also observed for 16α-hydroxylation of testosterone and androstenedione by 85–95% in microsomes from phenobarbital- and Aroclor 1254-treated rats. Our results clearly indicate that the 6β-hydroxylation of testosterone is catalyzed by an unidentified cytochrome P-450 isoform(s). Taken together, these results indicate that for some positions on the two steroids, most if not all of the metabolism catalyzed by microsomes is determined by a single P-450 isoform, while more than one cytochrome P-450 isoform must be involved in hydroxylations at other positions.

The marked positional specificity of C19 steroid hydroxylation by the five cytochrome P-450 isoforms reported above suggests that substrate binding sites of the hemoproteins have differing, but strict and well defined constraints. Recent studies evaluating the enantiomeric composition of the polycyclic aromatic hydrocarbon metabolites formed by cytochrome P-450c have provided a stereochemical model for the catalytic site of this hemoprotein (cf. Ref. 15). The regio- and stereospecific 6β-hydroxylation of both testosterone and androstenedione by cytochrome P-450c is consistent with the active site model. Interestingly, metabolism of the planar polycyclic hydrocarbons was associated with a high degree of stereoselectivity but not regioselectivity (27). The relatively inefficient metabolism of the polycyclic hydrocarbon benzo[a]pyrene by the other four cytochrome P-450 isoforms suggests the need for additional substrates to define the catalytic boundaries of these enzymes. The high positional specificity of the isoforms toward testosterone and androstenedione may make the steroids attractive candidates for such studies.

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Note Added in Proof—Since the submission of this manuscript, we have re-evaluated the effect of antibody directed against cytochrome P-450a on the metabolism of testosterone by hepatic microsomes from control and inducer-treated rats. While a previous study (3) utilizing a paper chromatography system for metabolite separation indicated that anti-P-450a partially (−50%) inhibited the 7α-hydroxylation of testosterone, we now find that the antibody totally inhibits this reaction in microsomes from untreated, phenobarbital-, 3-methylcholanthrene-, and Aroclor 1254-treated rats. We conclude that the resolution of 7α-hydroxytestosterone from other microsomal metabolites of testosterone was incomplete in the particular experiments described in the previous study (3). The only other microsomal metabolite whose formation was inhibited by the antibody was 6α-hydroxytestosterone. Utilization of a new plotting/reporting integrator with a wide signal range has enabled us to demonstrate that the purified monoxygenase system reconstituted with cytochrome P-450a catalyzes the 6α-hydroxylation of testosterone at approximately 5% of the rate for 7α-hydroxylation and that anti-P-450a completely inhibits both reactions.

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Steroid Metabolism by Cytochrome P-450 Isozymes

Regio- and stereoselective metabolism of two C19 steroids by five highly purified and reconstituted rat hepatic cytochrome P-450 isozymes.
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