on the properties and significance of the 90K protein beyond an involvement in steroid hormone action.

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Multiple Sites of Steroid Hydroxylation by the Liver Microsomal Cytochrome P-450 System: Primary and Secondary Metabolism of Androstenedione†

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ABSTRACT: To investigate the potential interaction of the various pathways of androgen hydroxylation, we have conducted studies to identify the profile of products formed during the time course of metabolism of androst-4-ene-3,17-dione (AD). Incubates containing AD, NADPH, and liver microsomes (from rats pretreated with phenobarbital) were sampled at times between 0 and 20 min and the metabolites resolved by reverse-phase (C_{18}) high-performance liquid chromatography. By this method, the pattern of formation and of utilization of eight major primary and secondary metabolites of AD was determined. We report here the formation of two previously unidentified major metabolites of AD: 6β,16α-dihydroxyandrost-4-ene-3,17-dione and 6β,16β-dihydroxyandrost-4-ene-3,17-dione. We propose that liver microsomal cytochromes P-450 can sequentially hydroxylate a single molecule of AD at multiple sites. These hydroxylase activities are presumably a result of multiple cytochrome P-450 isozymes acting on AD resulting in a transient time course for the appearance of some monohydroxylated metabolites. In addition, a unidirectional conversion of the metabolite 16α-hydroxyandrost-4-ene-3,17-dione to 16β-hydroxyandrost-4-ene-3,17-dione is described. Evidence is provided to support the role of cytochrome P-450 in catalyzing this reaction.

The cytochromes P-450 associated with the microsomal fraction of liver catalyze the NADPH- and oxygen-dependent metabolism of a variety of lipophilic foreign and endogenous compounds (Conney et al., 1968a, Cooper et al., 1979). It is generally considered that steroids and fatty acids constitute endogenous substrates of liver microsomal cytochromes P-450 (Conney et al., 1965; Kupfer, 1982). Studies with androst-4-ene-3,17-dione (AD)[1] or testosterone have shown that site-specific hydroxylation reactions are catalyzed by unique liver microsomal cytochromes P-450. These can occur at the

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[1] Abbreviations: HPLC, high-performance liquid chromatography; GC, gas chromatography; testosterone, 17β-hydroxyandrost-4-en-3-one; AD, androst-4-ene-3,17-dione; *X*αOH-AD, *X*α-hydroxyandrost-4-ene-3,17-dione, where *X* indicates the number of the hydroxyl-substituted carbon atom; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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6β, 7α, 16α, and 16β positions of the steroid molecule (Conney & Klutch, 1963, Nakamura & Ueda, 1980a,b). In addition, testosterone has been shown to be hydroxylated also at the 2α, 2β, and 15α positions (van der Hoeven, 1981, Conney & Klutch, 1963; Shiverich & Neims, 1979; Harada & Negishi, 1984). It is believed that the physiological importance of these reactions is to convert the steroid molecule into more polar products, susceptible to conjugation, and ultimately to facilitate its transport for excretion. However, possible biological activities of these stereospecifically hydroxylated steroids have not been fully explored.

The relative amounts of these different metabolites, determined by isolation and characterization after the in vitro incubation of either AD or testosterone with NADPH, oxygen, and liver microsomes, can be altered markedly by prior treatment of animals with various compounds known to modify the types and amounts of cytochrome P-450 isozymes associated with liver microsomes. Phenobarbital, pregnenolone-16α-carbonitrile, and testosterone propionate are examples of inducer compounds commonly used (Conney & Klutch, 1963; Lu et al., 1972b; Einarsson et al., 1973). The expression of certain liver microsomal steroid hydroxylase activities, such as the 16α-hydroxylase, has also been shown to be under developmental and hormonal regulation in the rat (Conney et al., 1968b; Levin et al., 1975; Waxman, 1984).

Using cytochromes P-450 isolated and purified from livers of rats pretreated with a variety of inducers, Lu et al. (1972a), Cheng & Schenkman (1982, 1983), Waxman et al. (1983, 1984), Ryan et al. (1983), and Wood et al. (1983) have recently demonstrated that individual cytochrome P-450 isozymes catalyze hydroxylation reactions at preferential sites on the steroid ring of testosterone and AD. Thus, it is now well established that the types of hydroxylated androgen metabolites formed during an in vitro incubation of the steroid with liver microsomes depend on the type and amount of the various cytochrome P-450 isozymes associated with the microsomal membrane. What is not understood are the molecular interactions between the cytochromes P-450 residing in the microsomal membrane, which may determine the ultimate profile of products formed during an in vitro incubation of steroids with liver microsomes. Possible interactions may include (1) secondary metabolism of primary metabolites, (2) competition of different P-450's for a limited supply of reducing equivalents supplied by the NADPH:cytochrome P-450 reductase, (3) the specific activity of each P-450 isozyme, (4) the relative affinity of the substrate (or product) to bind to each particular P-450 isozyme, and (5) the presence of other microsomal steroid-metabolizing enzymes, other than monooxygenases, which may compete for a limited amount of substrate.

As the first step in a series of studies designed to investigate these interactions, we have determined the time course of product(s) formation during the metabolism of AD by using liver microsomal preparations from male rats pretreated with the cytochrome P-450 inducer phenobarbital. By these studies we demonstrate the transient profile of metabolites formed during AD metabolism. These time-dependent metabolite profiles allow one to monitor the ability of the microsomal cytochromes P-450 to convert the initially formed monohydroxylated metabolites to dihydroxylated metabolites. These studies have revealed the identity of two major multihydroxylated metabolites of AD: the 6β,16α-dihydroxyandrost-4-ene-3,17-dione and the 6β,16β-dihydroxyandrost-4-ene-3,17-dione, not previously reported. In addition, a cytochrome P-450 dependent reaction is described that converts 16α-hydroxyandrost-4-ene-3,17-dione, a major metabolite of AD formed by liver microsomal cytochrome P-450, to 16β-hydroxyandrost-4-ene-3,17-dione.

Materials and Methods

Steroid Standards and Chemicals. 2α-, 2β-, 6α-, 6β-, 7α-, and 16α-hydroxyandrost-4-ene-3,17-diones and 3β,16β-dihydroxyandrost-5-en-17-one were supplied by Drs. Johnson and Kirk of the MRC Steroid Reference Collection, London. In addition, larger amounts of 6β- and 16α-hydroxyandrost-4-ene-3,17-diones, 3β,16β-dihydroxyandrost-5-en-17-one, and androst-4-ene-3,17-dione were obtained from Steraloids, Inc (Wilton, NH). [4-^{14}C]Androst-4-ene-3,17-dione (sp act. 59 mCi/mmol) was from Amersham (Arlington Heights, IL). High-performance liquid chromatography was performed on a Waters programmable HPLC using a 10μBondapack reverse-phase (C_{18}) column fitted with a precolumn filter. 3β-Hydroxysteroid dehydrogenase (EC 1.1.1.51) was purchased from Sigma (St. Louis, MO). Sep-Pac C_{18} cartridges and 0.45-μm filters were purchased from the Millipore Corp. (Bedford, MA). All other chemicals were of the highest purity available from commercial supply houses.

Preparation of Microsomes. Male Sprague-Dawley rats (150–200 g) were given a single intraperitoneal injection of phenobarbital (80 mg/kg, dissolved in water) followed by inclusion of 0.05% phenobarbital in their drinking water for 4 days. Control animals received no drugs. The animals were allowed food and water ad lib., but were starved overnight, prior to sacrifice by decapitation. Livers were perfused in situ with 0.9% sodium chloride, pooled, and homogenized in 5 volumes of 0.25 M sucrose with a Teflon pestle tissue homogenizer. The microsomal fraction was isolated by differential centrifugation as described by Remmer et al. (1966). Protein concentrations were determined by the biuret method (Gornall et al., 1949). Cytochrome P-450 content was determined spectrophotometrically by the method of Omura & Sato (1964). The specific content of cytochrome P-450 contained in various preparations of liver microsomes ranged from 2.6 to 3.5 and from 0.7 to 0.9 nmol of cytochrome P-450/mg of protein for microsomes obtained from livers of phenobarbital-pretreated and control animals, respectively.

Metabolism of Androstenedione. Rat liver microsomes were suspended in a reaction mixture consisting of 50 mM Tris-HCl buffer (pH 7.5), 150 mM KCl, 10 mM $MgCl_2$, 8 mM sodium isocitrate, and 0.5 unit/mL isocitrate dehydrogenase. [4-^{14}C]-Androst-4-ene-3,17-dione (sp act. 1.35 mCi/mmol) was added from a 20 mM solution in ethanol to give a final concentration of 100 μM. The mixture was preincubated at 37 °C for 1 min and the reaction initiated by the addition of NADPH to give a final concentration of 0.5 mM. The reaction mixture was magnetically stirred in a vessel open to air. At various time intervals, aliquots (2 mL) were removed and pipetted into a test tube containing methylene chloride (5 mL), vortexed, and placed on ice to stop the reaction. One milliliter of a saturated solution of NaCl was added, and the mixtures were extracted 3 times with methylene chloride (5 mL). Some samples that were incubated for longer periods of time were found to contain 5–10% of the initial counts remaining in the aqueous phase after extraction. This radioactivity could not be removed by further extractions with methylene chloride. These samples were evaporated to dryness by rotary evaporation, and the dried residue, suspended in methanol, was passed through a Sep-Pac C_{18} cartridge preequilibrated with methanol. The methanolic filtrate, which contained the residual counts, was then added to the original methylene chloride fraction. The overall extraction efficiency was de-

termined to be no less than 99%. The organic extract was dried with Na_2SO_4, filtered through a 0.45-μm filter, and evaporated to dryness at 30 °C under a stream of nitrogen. The residue containing the steroid metabolites was dissolved in 100 μL of methanol, and 50 nmol was analyzed by reverse-phase (C_{18}) HPLC using a linear gradient of 20–45% aqueous acetonitrile for 40 min followed by isocratic chromatography at 45% acetonitrile for 14 min at a flow rate of 1 mL/min (solvent system 1). In some cases, the metabolites were resolved by isocratic chromatography using methanol/water (1:1) (solvent system 2). The elution of metabolites was monitored spectrophotometrically at 254 nm and by determination of radioactivity in collected fractions (0.3 mL) by scintillation counting.

Isolation and GC–Mass Spectrometry Analysis of Androstenedione Metabolites. Sufficient concentrations of metabolites of AD were obtained from 25-mL reaction mixtures consisting of 100 μM [4-^{14}C]AD with 1 mg/mL of liver microsomal protein incubated at 37 °C for either 5 or 10 min in the presence of 0.5 mM NADPH as described above. After extraction with methylene chloride, the metabolites were separated by repetitive HPLC on an analytical reverse-phase (C_{18}) column and the appropriate fractions collected. Each metabolite fraction was extracted into ethyl acetate containing 0.01% BHT, dried with Na_2SO_4, filtered, and evaporated under a stream of argon. The metabolites were dissolved in methanol, quantitated by measurement of radioactivity and absorbance at 254 nm on HPLC, and stored at 4 °C under argon until further analysis or use as substrates.

Electron-ionization mass spectrometry was performed by either direct-inlet probe or by gas chromatographic inlet with a Finnigan 4021 GC/MS/DS equipped with a 6-ft column containing 3% QF-1 on gas chrom Q (Supelco). The injection port was set at 200 °C with a helium flow rate of 20 mL/min. The initial column temperature was 200 °C and increased at a rate of 5 °C/min to a maximum temperature of 280 °C, where chromatography was continued isothermally for an additional 10 min. All steroids were analyzed as their free hydroxyl or acetylated derivatives.

Acetylation of Steroids. Authentic reference samples and the isolated metabolites of AD were acetylated for further confirmation of identity with acetic anhydride and pyridine at a 1:1 molar ratio by the method of Dominguez et al. (1963). All the reactions attained 100% completion as determined by HPLC and GC analysis.

Synthesis of 16β-Hydroxyandrost-4-ene-3,17-dione. The synthesis was performed enzymatically by employing 3β-hydroxysteroid dehydrogenase (3β-HSD) from *Pseudomonas testosteroni*. This enzyme, in the presence of β-NAD, converts the Δ^5-3β-hydroxy group of a variety of steroids to a Δ^4-3-keto group (Talalay & Dobson, 1953). Briefly, 100 nmol of 3β,16β-dihydroxyandrost-5-en-17-one was dissolved in 0.1 M sodium phosphate, pH 8.9, containing 0.1 unit/mL 3β-HSD. In the absence of β-NAD, extraction of the reaction mixture yielded only one compound, corresponding to the substrate, which when separated by HPLC showed no absorbance at 254 nm but could be detected by GC analysis. After incubation for 3 min at 25 °C in the presence of β-NAD (0.2 mM), a single steroid metabolite was formed at a rate of 0.29 nmol min^{-1} (unit of enzyme)$^{-1}$. This steroid was extracted with methylene chloride, separated by HPLC, and shown to have absorbance at 254 nm, indicating the presence of a Δ^4-3-keto function. It did not correspond to 16-ketotestosterone since the acetylated derivatives of these two steroids could be separated by HPLC using solvent system 1. The compound has a shorter retention time on HPLC than 16α-hydroxyandrost-4-ene-3,17-dione but has the equivalent mass spectrum, thus confirming its identity as 16β-hydroxyandrost-4-ene-3,17-dione. The validity of this technique was confirmed by the synthesis of 16α-hydroxyandrost-4-ene-3,17-dione and androst-4-ene-3,17-dione from their corresponding Δ^5-3β-hydroxy analogues by 3β-HSD with the identical procedure described above for the synthesis of 16β-hydroxyandrost-4-ene-3,17-dione.

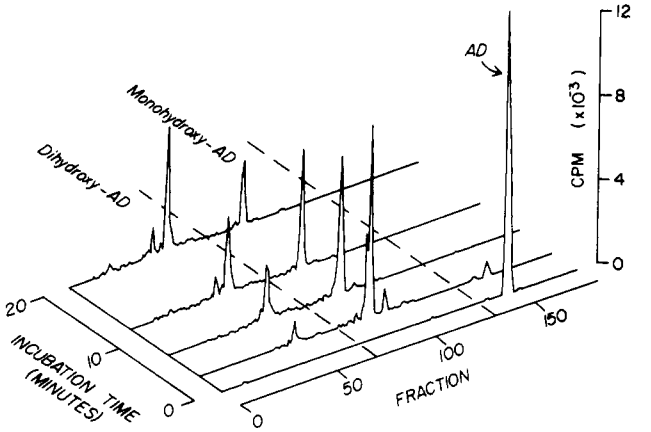

FIGURE 1: Profile of metabolites of androstenedione formed during oxidative metabolism by microsomal cytochromes P-450. Liver microsomes (1 mg of protein/mL) from male rats pretreated with phenobarbital were incubated for 0, 3, 7, 12, or 20 min in the presence of [4-^{14}C]androstenedione (100 μM) and NADPH as described under Materials and Methods. The metabolites (50 nmol) were resolved by HPLC, and 180 fractions of 0.3 mL each were collected for measuring radioactivity. The broken lines separate the relative areas where the different classes of hydroxylated metabolites elute.

RESULTS

Androstenedione Metabolism. Our initial studies were to characterize the different metabolites formed during the metabolism of AD by rat liver microsomes and to measure the time course of their formation. These studies were performed on liver microsomes prepared from male rats pretreated with phenobarbital, since these preparations of microsomes hydroxylate steroids at a rapid rate and produce metabolite profiles that are highly reproducible when the results obtained from different preparations are compared. A HPLC system was optimized to separate all known metabolites of AD and to observe the formation and subsequent utilization of as yet unidentified metabolites. The presence of each metabolite produced was monitored spectrophotometrically at 254 nm and by the amount of radioactivity contained in appropriate fractions. These parameters were also used to help identify and quantify each metabolite.

An example of the profile of metabolites obtained following an incubation of [4-^{14}C]AD (100 μM) for various times with liver microsomes is shown in Figure 1. Under these assay conditions, eight major metabolites have been separated, identified, and observed to be formed at a rate of at least 0.5 nmol min^{-1} (mg of protein)$^{-1}$ during an incubation of AD with liver microsomes prepared from rats pretreated with phenobarbital. These eight metabolites constitute at least 85% of all the metabolites formed during the first 5–10 min of incubation. Seven exhibit absorbance at 254 nm, indicating retention of the Δ^4-3-keto function on the parent steroid. Less than 2% of the AD was converted to 5α-androstanedione, indicating that under the assay conditions employed there is a relatively low amount of microsomal 5α-reductase activity active on AD associated with liver microsomes isolated from

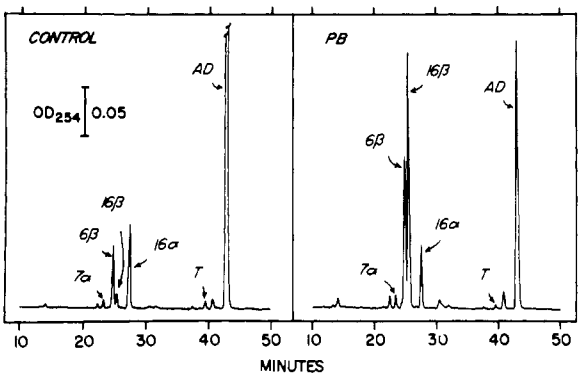

FIGURE 2: Profile of metabolites of androstenedione resolved by HPLC. (Left) Absorbance tracing of metabolites formed after incubating liver microsomes (0.25 mg of protein/mL) from nontreated male rats with androstenedione (100 μM) for 5 min at 37 °C in the presence of NADPH (0.5 mM) and a NADPH-generating system. (Right) The identical experiment as on the left except using liver microsomes from rats pretreated with phenobarbital. The steroid metabolites were detected by their absorbance at 254 nm and are labeled according to the site of the hydroxyl-substituted carbon atom. AD and T represent androstenedione and testosterone, respectively.

male rats pretreated with phenobarbital. Initial analysis of the mass spectra of the metabolites showed that they are retained on the reverse-phase HPLC column in such a manner that they can be grouped into two broad categories: (1) the monohydroxylated and (2) dihydroxylated androstenediones, as demonstrated in Figure 1. A general pattern of formation can be seen whereby the monohydroxylated metabolites are rapidly formed but then decrease in content. The dihydroxylated metabolites, on the other hand, form more slowly but continue to increase in content, suggesting that sequential hydroxylation of the parent steroid is occurring.

Identification of the Monohydroxylated Metabolites of Androstenedione. Identification of some of the monohydroxylated metabolites of AD was made by comparison of their retention times with known standards on HPLC and GC and by their mass spectra. In addition, the primary metabolites were acetylated, and their retentions on HPLC and GC were compared to those of acetylated authentic standards. Metabolites identified from available standards include 6β-, 7α-, and 16α-hydroxyandrost-4-ene-3,17-diones and testosterone, thus demonstrating both steroid hydroxylase and 17-oxidoreductase activities present in the microsomes (see Figure 2). The most abundant metabolite formed during the oxygenation of AD by enzymes present in liver microsomes from rats pretreated with phenobarbital is 16β-hydroxyandrost-4-ene-3,17-dione. As shown in Figure 2 and as shown by others (Nakamura & Ueda, 1980a; Wood et al., 1983), the formation of 16β-hydroxyandrost-4-ene-3,17-dione is stimulated about 20-fold when the initial rates from male rats pretreated with phenobarbital relative to nontreated animals are compared.

Time Course of Product Formation. To investigate the time course for the formation of the various metabolites of AD, a determination of metabolite concentration at various time intervals during an incubation of [4-^{14}C]AD with various concentrations of rat liver microsomes was made. The results are plotted in Figure 3. The total content of AD in the reaction mixture is oxidatively metabolized with time, indicating that the added substrate is completely accessible to the hydroxylase enzymes associated with the microsomal membrane. The formation of 6β-, 16α-, and 16βOH-AD's is reflected by an initial rapid rise in their concentration followed by a slower steady decline. As predicted, the duration of this time course is dependent both on the concentration of liver microsomes (i.e., cytochrome P-450) present in the reaction

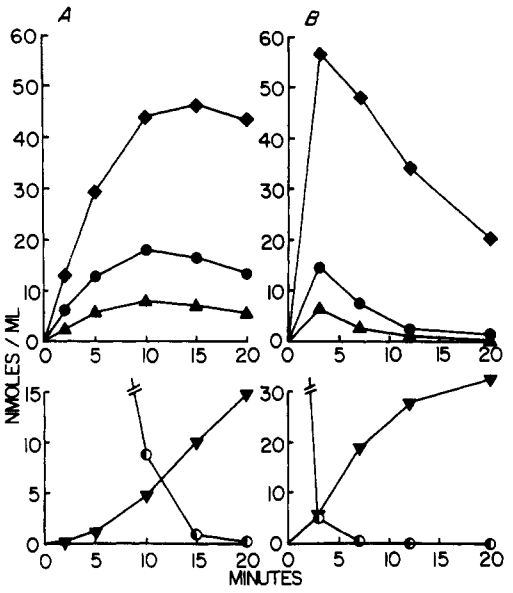

FIGURE 3: Time course for the formation of metabolites of androstenedione. [4-^{14}C]Androstenedione (100 μM) was incubated for various times at 37 °C in the presence of NADPH and (A) 0.25 or (B) 1.0 mg/mL liver microsomal protein from male rats pretreated with phenobarbital. Metabolites were separated by HPLC and quantitated by liquid scintilation counting. (▲) 16αOH-AD; (●) 6βOH-AD; (♦) 16βOH-AD; (◐) AD; (▼) 6β,16αOH-AD plus 6β,16βOH-AD.

mixture (compare Figures 3A and 3B) and on the initial concentration of AD used as substrate (data not shown). This multiphasic time course indicates that these monohydroxylated metabolites of AD are being further metabolized to form "secondary" metabolites. The concentration of any particular monohydroxylated metabolite determined at a given point in time, as shown in Figure 3, therefore represents the difference between the amount which is formed and the amount which is subsequently metabolized to form a secondary metabolite.

Formation of Multihydroxylated Metabolites. A group of metabolites having retention times between 10 and 15 min on HPLC was observed to accumulate at a steady rate after experiencing a lag period lasting up to 2 min, depending upon the amount of protein and/or substrate present during an incubation of [4-^{14}C]AD with rat liver microsomes. These compounds could be detected by absorbance at 254 nm, which indicated that they were not reductive metabolites of AD. The presence of a lag period before their formation suggests that these compounds may represent secondary metabolites of AD formed by the further oxidation of the monohydroxylated metabolites. Further evidence for the identification of these polar compounds as dihydroxylated metabolites of AD came from the observations that (1) the mass spectra of these compounds exhibit a molecular ion at *m/e* 318, (2) the rate of production of these polar compounds during the later stages of the incubation period (15–20 min) exceeds the rate of AD being metabolized during this interval (see Figure 3), and (3) the production of these compounds is observed to continue even after the concentration of AD in the reaction mixture has reached zero, (see Figures 1 and 3), thus demonstrating that these metabolites are not derived directly from AD.

Figure 4 shows that the major secondary metabolite formed following an incubation of AD with liver microsomes for 20 min, eluted as a fraction having a retention time of 14 min on HPLC in solvent system 1. To identify this compound, it was purified from the other metabolites by HPLC and then further resolved into two peaks in solvent system 2 (see Figure 4). These two compounds were separated by HPLC in 43%

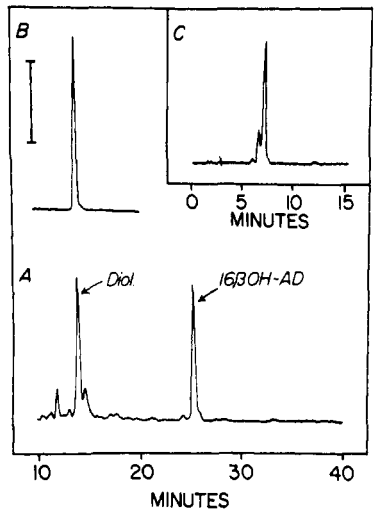

FIGURE 4: Characterization of the major secondary metabolites of androstenedione. (A) Profile of the metabolites resolved by HPLC in solvent system 1 after a 20-min incubation of AD (100 μM) with liver microsomes (1 mg of protein/mL) from male rats pretreated with phenobarbital. (B) HPLC chromatogram of the major secondary metabolite of AD (labeled diol in panel A) purified by HPLC in solvent system 1. (C) HPLC chromatogram of the purified metabolite shown in panel B in solvent system 2. Bar equals 0.05 OD at 254 nm.

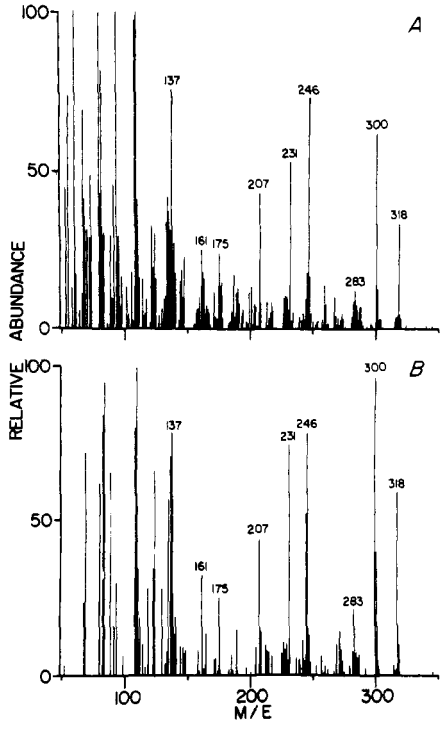

FIGURE 5: Mass spectra of the major secondary metabolites of AD. (A) Mass spectrum of the compound tentatively identified as 6β,16α-dihydroxyandrost-4-ene-3,17-dione, representing the first peak (retention time 6.7 min) in Figure 4C. (B) Mass spectrum of the compound tentatively identified as 6β,16β-dihydroxyandrost-4-ene-3,17-dione, representing the second peak (retention time 7.2 min) in Figure 4C.

aqueous methanol. Figure 5 shows that when these two separated fractions were analyzed by mass spectrometry, they were discovered to have an equivalent fragmentation pattern, suggesting that these dihydroxylated metabolites of AD may be epimers of each other. A clue to the identity of these two metabolites came from the observation that when AD was incubated with a low concentration of liver microsomes (0.25 mg of protein/mL), the rate of formation of these two dihydroxylated metabolites approximated the sum of the rates

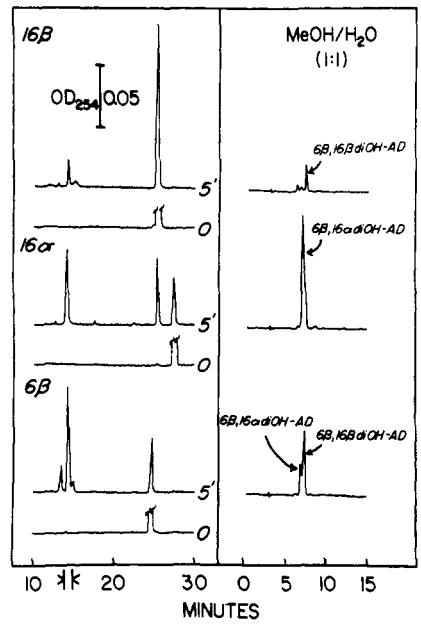

FIGURE 6: Metabolism of 16β-, 16α-, or 6β-hydroxyandrost-4-ene-3,17-dione by rat liver microsomal cytochromes P-450. Either [4-^{14}C]-16β-, [4-^{14}C]-16α-, or [4-^{14}C]-6β-hydroxyandrost-4-ene-3,17-dione (0.3 mCi/mmol) at 50 μM was incubated with liver microsomes (1 mg of protein/mL) from rats pretreated with phenobarbital. Products were resolved by HPLC at 0 (before the addition of NADPH) and at 5 min after the addition of NADPH. The rates were 2.5, 8.4, and 6.7 nmol min^{-1} (mg of protein)$^{-1}$ for the metabolism of 16β-, 16α-, and 6βOH-AD's, respectively. The chromatogram on the left side of the figure represents the results obtained in solvent system 1. The chromatogram on the right side of the figure represents the metabolites collected between 13.5 and 14.5 min in each experiment in solvent system 1 and further resolved by HPLC in solvent system 2.

of disappearance of 6β-, 16β- and 16αOH-AD during the later stages of the incubation (see Figure 3A). This suggested that these two compounds may be secondary metabolites derived from the further metabolism of 6β-, 16β-, and 16αOH-AD. To test this, we incubated 4-^{14}C-labeled 6β-, 16α-, or 16βOH-AD separately with liver microsomes from rats pretreated with phenobarbital and then separated the metabolites by HPLC. Figure 6 (left panel) presents the HPLC profiles before and after a 5-min incubation in the presence of NADPH. In each of the three cases, a major dihydroxylated metabolite was obtained in good yield whose retention time of 14 min on HPLC in solvent system 1 corresponded to the major dihydroxylated metabolites formed during an incubation of liver microsomes with AD. Further chromatography of this dihydroxy metabolite fraction, formed following the metabolism of [4-^{14}C]6βOH-AD, in solvent system 2, showed the fraction to consist of two compounds whose mass spectra corresponded to the two apparent epimers formed from AD shown in Figure 4C. Chromatography of the metabolites formed following the metabolism of 16α- or 16βOH-AD in solvent system 2, however, showed each to contain only a single major epimer. These results lead to the conclusion that the two epimeric metabolites formed from 6βOH-AD are 6β,16β-diOH-AD and 6β,16α-diOH-AD, whereas that formed from 16αOH-AD is 6β,16α-diOH-AD and that formed from 16βOH-AD is 6β,16β-diOH-AD. Thus it appears that liver microsomes from rats pretreated with phenobarbital are able to hydroxylate a single steroid molecule sequentially at multiple sites to form metabolites that are very polar in physical character.

Conversion of 16α-Hydroxyandrost-4-ene-3,17-dione to 16β-Hydroxyandrost-4-ene-3,17-dione. Analysis of the re-

Table I: Characterization of the Rat Liver Microsomal Reaction That Converts 16α-Hydroxyandros-4-ene-3,17-dione to 16β-Hydroxyandrost-4-ene-3,17-dione

condition[a]	16β-hydroxyandrostenedione [nmol min^{-1} mg of protein)$^{-1}$]
complete system	2.9
without NADPH	0.0
NADH[b]	0.1
heat treated[c]	0.0
+0.2 mM metyrapone	0.2
+10 μM clotrimazole	0.0
+80:20 CO/O_2	0.6

[a] [4-^{14}C]16αOH-AD (50 μM, sp act. 0.6 nCi/nmol) was incubated with rat liver microsomes (protein, 1 mg/mL), 8 mM sodium isocitrate, 0.5 unit/mL isocitrate dehydrogenase, and NADPH (0.5 mM) (complete system) for 5 min at 37 °C. The conversion of 16αOH-AD to 16βOH-AD was monitored by HPLC and quantitated by liquid scintillation counting. [b] NADH (0.5 mM) was substituted for NADPH. [c] Heat treatment consisted of warming the microsomal protein suspension to 90 °C for 2 min and then cooling to room temperature prior to use in the assay.

action products shown in Figure 6, obtained following the incubation of [4-^{14}C]16αOH-AD with rat liver microsomes, showed the production of an additional metabolite (retention time of 25 min in solvent system 1) at a rate of 2.9 nmol min^{-1} (mg of protein)$^{-1}$ that did not chromatograph as a dihydroxylated metabolite. Further characterization of this metabolite and its acetylated derivative by HPLC and GC/mass spectrometry revealed its identity as 16βOH-AD (see Figure 7).[2] As shown in Table I, the conversion of 16αOH-AD to 16βOH-AD depends upon the presence of NADPH and native microsomal protein and is inhibited by the presence of CO or by substituted phenylimidazoles, implicating a role of cytochrome P-450. Interestingly, no isomerization of 16βOH-AD to form 16αOH-AD was observed (see Figure 6), demonstrating that this rat liver microsomal reaction apparently proceeds in essentially only one direction (α → β).

Discussion

Reactions catalyzed by cytochromes P-450 play a central role in the synthesis and degradation of steroids, starting from the synthesis of cholesterol, its subsequent conversion to steroid hormones and bile acids, and the later metabolism of these compounds by the liver. It is generally believed that the cytochromes P-450 involved in the synthesis of steroids are highly specific in their substrate preference and site of hydroxylation. Liver microsomal cytochromes P-450 also catalyze stereo- and regiospecific hydroxylations of steroids, although individual purified P-450 isozymes have also been shown to metabolize a variety of xenobiotics in addition to their hydroxylation of steroids (Harada & Negishi, 1984; Ryan et al., 1982). The activity of the various steroid hydroxylase reactions occurring in the liver can be altered by pretreatment of animals with compounds such as phenobarbital, which change the content of cytochromes P-450 associated with the microsomal membrane (Conney, 1967). The results of this study and of other workers (Nakamura & Ueda, 1980a; Wood et al., 1983) show that for the substrate AD the major steroid hydroxylase activity induced by phenobarbital pretreatment is the 16β-hydroxylase. This activity is stimulated by approximately 20-fold over that of nontreated animals and accounts for the majority of the increase in the in vitro measurement of AD metabolism when liver microsomes from phenobarbital-treated rats are used.

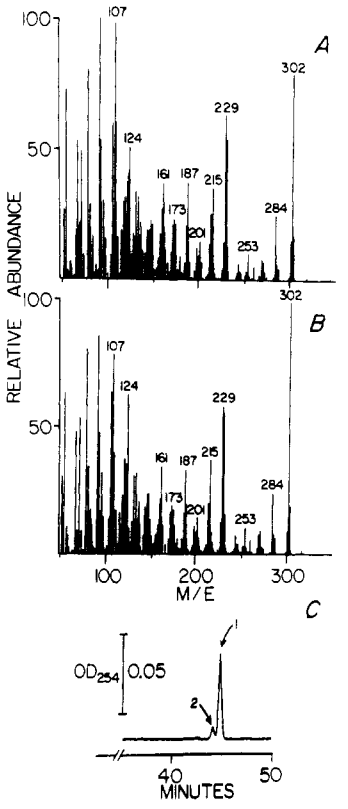

FIGURE 7: Characterization of the reaction product formed from 16α-hydroxyandrost-4-ene-3,17-dione. (A) Mass spectrum of the compound having a retention time of 25 min on HPLC in solvent system 1 obtained after incubating 16αOH-AD with rat liver microsomes. (B) Mass spectrum of 16β-hydroxyandrost-4-ene-3,17-dione. (C) HPLC chromatogram of the acetylated reaction product in solvent system 1. Arrow number 1 indicates where 16β-hydroxyandrost-4-ene-3,17-dione acetate elutes, and arrow number 2 indicates where 16-ketotestosterone acetate elutes.

In addition to the production of monohydroxylated steroid metabolites, the results presented in this study suggest that there exists a pathway for sequential hydroxylation of steroids in liver microsomes. We have identified two major dihydroxylated metabolites of androstenedione as 6β,16β-dihydroxyandrost-4-ene-3,17-dione and 6β,16α-dihydroxyandrost-4-ene-3,17-dione. Earlier studies by Jacobson et al. (1969) presented evidence that rat liver microsomal cytochromes P-450 have the ability to metabolize the monohydroxylated metabolites of testosterone to produce more polar compounds, although the authors did not identify these metabolites. The ability of testosterone to competitively inhibit this secondary metabolism suggested that the same enzymes functioning to produce the "primary" metabolites may also be acting to form the "secondary" metabolites. Lisboa et al. (1968) tentatively identified a dihydroxylated metabolite of testosterone formed by rat liver microsomes as 6β,16α,17β-trihydroxyandrost-4-en-3-one. Incubating testosterone with purified cytochrome P-450b in a reconstituted system, Wood et al. (1983) demonstrated that this individual isozyme has the capacity to sequentially convert testosterone first to AD and then to 16βOH-AD. However, the sequential oxidation of testosterone to form dihydroxylated testosterone derivatives could not be catalyzed by any one of the five individual P-450 isozymes tested, indicating that this type of reaction may be dependent upon the action of more than one cytochrome P-450

[2] The small amount of 16-ketotestosterone acetate present is believed to be formed from the metabolic product 16β-hydroxyandrost-4-ene-3,17-dione during the base-catalyzed acetylation reaction. 16β-Hydroxy-17-keto steroids have been shown to undergo rearrangement to form 17β-hydroxy-16-keto steroids in the presence of strong acid or base (Waxman et al., 1983).

isozyme in a unique orientation provided by the membrane.

The transfer of hydroxylated steroid metabolites from one liver microsomal cytochrome P-450 to another and the possible ways that this process may be regulated have not been fully explored. It is not known whether this process requires a transmembrane transport of the primary metabolite to a different cytochrome P-450 for further oxidation or whether the two isozymes reside juxtaposed in the same membrane and the dihydroxylated metabolite is formed by a P-450 to P-450 transfer of the steroid within the same membrane. Resolution of this problem is central to understanding the regiopositioning of the various steroid hydroxylases within the membrane of the endoplasmic reticulum.

Studies of the hydroxylation of 16αOH-AD to form 6β,16α-diOH-AD by liver microsomal cytochromes P-450 resulted in the unexpected observation that 16αOH-AD is also efficiently converted to 16βOH-AD. This reaction is shown to depend on the presence of native microsomal protein and NADPH and is inhibited by the inclusion of either CO or substituted phenylimidazoles, thus supporting the conclusion that a cytochrome P-450 plays a role in the reaction. The ability of liver microsomal cytochrome P-450 to catalyze this type of reaction during the metabolism of steroids has not previously been reported. Further studies are required to characterize the mechanism of this reaction with respect to the origin of the oxygen atom of the hydroxyl group, a determination of which cytochrome P-450 isozyme is involved, and the relative significance of this pathway in the overall production of 16βOH-AD from AD.

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Registry No. AD, 63-05-8; 6β,16αdiOH-AD, 98361-36-5; 6β,16βdiOH-AD, 98361-37-6; 16αOH-AD, 63-02-5; 16βOH-AD, 59685-30-2; 6βOH-AD, 63-00-3; 7αOH-AD, 62-84-0; cytochrome P-450, 9035-51-2; testosterone, 58-22-0.

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