Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 9756–9760, December 1990
Biochemistry

Characterization of the steroid-metabolizing capacity of the hepatic cytochrome P450IIC5 expressed in COS-1 cells: 3β-Hydroxysteroid dehydrogenase/$\Delta^{5\rightarrow4}$ isomerase type activity

(rabbit/liver/21-hydroxylase/16α-hydroxylation)

JOHN M. TRANT*†, MATTHEW C. LORENCE*‡, ERIC F. JOHNSON§, CEDRIC H. L. SHACKLETON¶, J. IAN MASON*, AND RONALD W. ESTABROOK*‖

*Department of Biochemistry and Cecil H. and Ida Green Center for Reproductive Biology Sciences, University of Texas Southwestern Medical Center, Dallas, TX 75235; §Division of Biochemistry, Department of Basic and Clinical Research, Scripps Clinic and Research Foundation, La Jolla, CA 92037; and ¶Children's Hospital Medical Center, Children's Hospital Research Laboratory, Oakland, CA 94609

Contributed by Ronald W. Estabrook, September 21, 1990

ABSTRACT **Cytochrome P450IIC5 (rabbit liver 21-hydroxylase) is unusual among hepatic forms of cytochromes P450 because it catalyzes the conversion of one active steroid hormone (progesterone) to another active hormone (deoxycorticosterone). Another interesting aspect of this steroid-hydroxylating enzyme is the ability to convert Δ^5-3β-hydroxysteroids to the Δ^4-3-ketosteroid configuration. The Δ^5-3β-hydroxysteroid, pregnenolone, was readily 21-hydroxylated, and this product was further metabolized to the Δ^4-3-ketosteroid, deoxycorticosterone. It is suggested that the mechanism of this cytochrome P450-mediated, 3β-hydroxysteroid dehydrogenase/$\Delta^{5\rightarrow4}$ isomerase-like reaction is through a *gem*-diol formation. In this study, COS-1 cells were transfected with the plasmid encoding cytochrome P450IIC5 to express a functional enzyme within the cell milieu. Transfected COS cells preferentially metabolize pregnenolone compared with all other steroids tested. Progesterone and 17α-hydroxypregnenolone are also 21-hydroxylated, whereas 17α-hydroxyprogesterone is a poor substrate. Substrate preference of this 21-hydroxylase differs from that seen with bovine adrenal P450XXIA1 (formerly $P450_{C21}$) hydroxylase. Additionally, this study demonstrated that C_{19} steroids, like dehydroepiandrosterone and androstenedione, are hydroxylated at the 16α position. Contrary to previous reports, no metabolite of estradiol-17β was detected, presumably due to the unstable nature of catechol estrogens (2-hydroxyestradiol).**

The multiplicity of microsomal cytochromes P450 has been clearly established by isolation and characterization. In the rabbit, at least seven distinct forms of P450 have been isolated (1, 2). These include form 2, which is the prinicipal form induced in the liver by phenobarbital (3, 4) and which represents one of two major forms in lung microsomes isolated from untreated rabbits (5, 6). Also associated with rabbit hepatic microsomes are forms 4 and 6, which are differentially induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in a tissue- and age-specific manner (7, 8). Forms 3*b* and 3*c* (9, 10), which are capable of 6β- and 16α-hydroxylation of progesterone (2), have also been isolated and characterized (11, 12). The enzyme of interest to this study is form 1, a hepatic steroid 21-hydroxylase (13). This enzyme is now designated as cytochrome P450IIC5, according to a proposed uniform system of nomenclature (14).

Cytochrome P450IIC5 is unusual among hepatic forms of cytochromes P450 because it metabolizes the conversion of progesterone to 11-deoxycorticosterone (DOC) at rates similar to those with cytochrome P450XXIA, the adrenal microsomal steroid 21-hydroxylase (15). Although hepatic steroid-metabolizing P450s typically inactivate steroid hormones, the metabolite DOC, formed from progesterone by P450IIC5, is a mineralocorticosteroid. In addition, P450IIC5 exhibits a high degree of genetic polymorphism, indicating its potential variability in contributing to steroid metabolism. In contrast to the cloned bovine adrenal 21-hydroxylase expressed in COS-1 cells (16), reconstituted microsomal preparations of P450IIC5 have also been shown to catalyze the 21-hydroxylation of pregnenolone (17) as well as the 2-hydroxylation of estradiol (18), the hydroxylation of benzo(α)-pyrene (19), and the 7-hydroxylation of 2-acetylaminofluorene (20).

Recently, P450IIC5 has been cloned (21) and expressed in COS-1 cells, a nonsteroidogenic cell line (22). Expression of cytochromes P450 in COS-1 cells has been a useful technique for the characterization of an enzyme's potential activity without interference of or contributions by other cytochromes P450 (refs. 16 and 23, for examples). Here we characterize the steroidogenic capacity of cytochrome P450IIC5 as a dehydrogenase-like enzyme that catalyzes the conversion of Δ^5-3β-hydroxysteroids to Δ^4-3-ketosteroids in addition to its ability to catalyze the 21-hydroxylation of progesterone and pregnenolone.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium was from GIBCO. Methanol and methylene chloride were of HPLC grade (Burdick and Jackson); Milli-Q purified water and the methanol were filtered (0.45 μm) before use in HPLC. [1,2,6,7-^{3}H]Progesterone (96 Ci/mmol; 1 Ci = 37 GBq), 17α-[1,2,6,7-^{3}H]hydroxyprogesterone (63 Ci/mmol), 17α-[7(n)-^{3}H]hydroxypregnenolone (12 Ci/mmol), [4,7-^{3}H]pregnenolone (12.7 Ci/mmol), [1,2-^{3}H]dehydroepiandrosterone (13 Ci/mmol), [4-^{14}C]androstenedione (59 mCi/mmol), and [4-^{14}C]estradiol-17β (55 mCi/mmol) were purchased from Amersham, freed of carrier solvent, dissolved in ethanol, and used without further purification. Dehydroepiandrosterone (DHEA) and 3β,4α-dihydroxy-5-androstene-17-one were

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: DOC, 11-deoxycorticosterone; 3β-HSD, 3β-hydroxysteroid dehydrogenase/$\Delta^{5\rightarrow4}$isomerase; DHEA, dehydroepiandrosterone.

†Present address: Department of Zoology and Physiology, Louisiana State University, Baton Rouge, LA 70803-1725.
‡Present address: Bio-Rad Laboratories, Molecular Biology Products Group, 15111 San Pablo Ave., Richmond, CA 94806.
‖To whom reprint requests should be addressed at: Department of Biochemistry, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235-9038.

purchased from Steraloids (Wilton, NH); all other steroids were purchased from Sigma.

COS-1 Cell Transfection. COS-1 cells were grown in DMEM/10% iron-supplemented calf bovine serum (HyClone) and transfected with 5 μg of plasmid DNA per ml of transfection medium in 60-mm or 100-mm dishes by using DEAE-dextran as described (23). The coding region of P450IIC5, formerly known as pl-8, was inserted into vector pCMV1 (24). DNA sequence analysis identified an additional initiation codon located 14 base pairs (bp) upstream and in a different reading frame from the true initiation codon of pl-8 cDNA. To prevent incorrect initiation of translation, a 23-bp *Cla* I fragment containing the upstream initiation codon was deleted to give pCMVIIC5, as described before (22). This construct contains the entire rabbit liver P450IIC5 cDNA coding region with the initiation codon located ≈74 bp from the cytomegalovirus promoter and was used for all subsequent COS-1 cell transfections.

Enzyme Assays. Radiolabeled steroid substrates (100,000 cpm per ml of medium) were added, along with nonradioactive substrate, to a final concentration of 1 μM (unless otherwise noted), 72 hr after transfection. Aliquots of medium (0.5 ml) were removed at various time intervals and extracted with 5 ml of methylene chloride, and the organic phase was evaporated to dryness at 40°C under a stream of nitrogen. The residue was dissolved in methanol and subjected to reverse-phase HPLC.

Chromatography. HPLC was conducted using a Waters 30-cm μBondapak C_{18} column. The solvent-delivery profile consisted of a 30-min methanol/water 60:40 elution, then a 15-min linear gradient to 100% methanol, and finally an additional 15 min with 100% methanol. Radioactive material was detected by an on-line liquid scintillation spectrometer (Radiomatic Flo-One; Meriden, CT). In instances where radioactive substrates were not available, UV 254-nm absorbance was used for detection and quantification.

TLC was conducted on silica-gel GF plates with preabsorbent zone (Analtech) and double developed in a saturated chamber of benzene/methanol 9:1. Radio-inert steroids were detected with UV 254-nm illumination, and tritiated steroids were detected with liquid scintillation spectrophotometry.

Steroids were analyzed as their methyl oxime, trimethylsilyl ether derivatives by gas chromatography and mass spectrometry. To some of the steroid extracts purified by HPLC three internal standards were added (5α-androstane-3α,17α-diol, stigmasterol, and cholesteryl butyrate), and methyl oxime derivatives were made of reactive carbonyl groups. This derivatization was achieved by adding 0.1 ml of 2% methoxyamine hydrochloride in pyridine, and the solution was incubated for 1 hr at 60°C. After drying under nitrogen, 50 μl of trimethylsilylimidazole was added, and silylation proceeded overnight at 100°C. The derivatized samples were separated on a 15-m SPB-1 fused-silica capillary column attached to a Hewlett–Packard 5970 mass spectrometer. The samples (dissolved in cyclohexane) were injected without splitting the stream with an oven temperature of 50°C. This temperature was held for 3 min; then the oven temperature was rapidly taken to a standing temperature of 230°C. The temperature was programmed to 310°C at 2°C/min. Mass spectra were obtained by repetitive scanning over the 100- to 800-atomic mass unit range.

RESULTS

Comparison of the Enzymatic Activities of Adrenal and Liver 21-Hydroxylases Expressed in COS-1 Cells. The rates of progesterone metabolism by rabbit liver 21-hydroxylase (P450IIC5) and bovine adrenal 21-hydroxylase (P450XXI) expressed in COS-1 cells are similar (Fig. 1), as was demonstrated with microsomal preparations (15). In both cases,

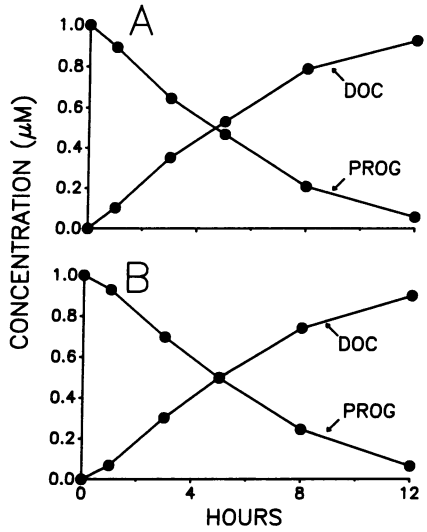

FIG. 1. (*A*) Time course of tritiated progesterone (1 μM) metabolism by COS-1 cells transfected with plasmid encoding P450IIC5. (*B*) Time course of tritiated progesterone (1 μM) metabolism by COS-1 cells transfected with plasmid encoding bovine adrenal P450XXI. Prog, progesterone. No metabolism of progesterone was noted in mock-transfected COS-1 cells.

only a single metabolite was isolated from the media. Similar experiments were conducted with various sizes of tissue-culture dishes (60 or 100 mm), various transfections (up to five experiments), and various substrate concentrations (1–5 μM). These data were used to construct Table 1. Table 1 lists the ratios of the rates of metabolism of a variety of substrates. The preferred substrate of the adrenal 21-hydroxylase, 17α-hydroxyprogesterone, is not readily metabolized by the hepatic 21-hydroxylase. Conversely, the Δ^5-pregnenes do not act as substrates for the adrenal enzyme; however, pregnenolone is the preferred substrate for the hepatic enzyme, and 17α-hydroxypregnenolone is metabolized as rapidly by the hepatic enzyme as progesterone.

The C_{19} steroids, DHEA and androstenedione, are also metabolized by the rabbit liver 21-hydroxylase, whereas there is no detectable metabolism of the steroids by the adrenal 21-hydroxylase. We were unable to detect any metabolites isolated from cells incubated with estradiol-17β, even though reconstituted, purified P450IIC5 has been demonstrated to readily hydroxylate this estrogen at the C-2 position (18). However, microsomes from transfected cells catalyzed the 2-hydroxylation of estradiol (data not shown). The rate is ≈15–20% the rate obtained for progesterone 21-hydroxylase activity when both substrates are present at an initial concentration of 20 μM. This rate is ≈10 times background. To isolate the catechol product, ascorbic acid must be included in the incubation and in the TLC systems (18).

Table 1. Comparison of rates of metabolism of various steroids by liver (P450IIC5) and adrenal (P450XXI) 21-hydroxylase

Steroid substrate	Enzymatic activity, rate ratio	
	Bovine adrenal	Rabbit liver
17α-Hydroxyprogesterone	1.0	0.08
Progesterone	0.4	0.4
Pregnenolone	ND	1.0
17α-Hydroxypregnenolone	ND	0.4
DHEA	ND	0.5
Androstenedione	ND	0.09
Estradiol-17β	ND	ND

Rates are presented as ratios; 1.0 represents the most rapid rate of substrate disappearance. ND, not detectable.

Various C-21 Products of Rabbit Liver 21-Hydroxylase. As demonstrated in Fig. 1, progesterone is hydroxylated at the C-21 position, resulting in the synthesis of a single product, DOC. Although not shown, 17α-hydroxyprogesterone is likewise hydroxylated to form a single product, 11-deoxycortisol.

In contrast, metabolism of Δ^5-steroids resulted in a variety of steroid products. Fig. 2 shows that pregnenolone (1 μM) is rapidly hydroxylated at the C-21 position. However, by a mechanism not yet understood, 21-hydroxypregnenolone is converted to 21-hydroxyprogesterone in a reaction similar to that catalyzed by 3β-hydroxysteroid dehydrogenase/$\Delta^{5\rightarrow4}$ isomerase (3β-HSD) (EC 1.1.1.145) (Fig. 2*A*). Although not illustrated, a trace quantity of progesterone was isolated from incubations with higher initial concentrations of pregnenolone (5 and 10 μM). The addition of radio-inert 10 μM 21-hydroxypregnenolone significantly reduced the synthesis of tritiated DOC (Fig. 2*B*). However, the accumulation of UV 254-nm absorbing material that coeluted with DOC was greatly enhanced. Fig. 3 illustrates the time course of accumulation of this UV 254-nm absorbing material derived from 0, 1, and 5 μM initial concentrations of 21-hydroxypregnenolone. This steroid was collected from incubations with 21-hydroxypregnenolone and pregnenolone and was tentatively identified as DOC due to its UV 254-nm absorbing characteristic and comigration with DOC standards on HPLC and TLC.

The 3β-HSD-Like Activity. The conversion of the Δ^5-3β-hydroxysteroids to Δ^4-3-ketosteroids is conventionally mediated by the enzyme 3β-HSD. However, mock-transfected COS cells demonstrated no 3β-HSD activity with pregnenolone, 17α-hydroxypregnenolone, or DHEA. This 3β-HSD-like activity is mediated by cytochrome P450IIC5 as shown in Fig. 4. Fig. 4*A* shows a time course of the metabolism of 1 μM tritiated pregnenolone. With addition of 10 μM clotrimazole, a general cytochrome P450 inhibitor (25, 26), the rate of pregnenolone utilization is decreased to half, but the accumulation of DOC is eliminated (Fig. 4*B*). The addition of trilostane at 2.5 μg/ml, a 3β-HSD-specific inhibitor, did not alter the rate of pregnenolone utilization and only partially inhibited the rate of DOC accumulation (Fig. 4*C*). This partial inhibition may be due, in part, to the ability of trilostane to inhibit metabolism of a substrate such as 21-hydroxypregnenolone, which probably has a higher K_m than pregnenolone (as shown in Fig. 4*C*).

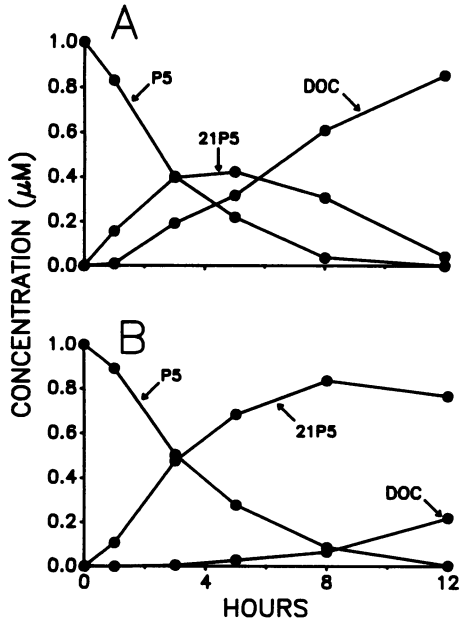

FIG. 2. Time course of tritiated pregnenolone (1 μM) metabolism by COS-1 cells transfected with plasmid encoding P450IIC5. P5, pregnenolone; 21P5, 21-hydroxypregnenolone. (*A*) Control. (*B*) Same as *A*, except supplemented with 10 μM of radio-inert 21-hydroxypregnenolone.

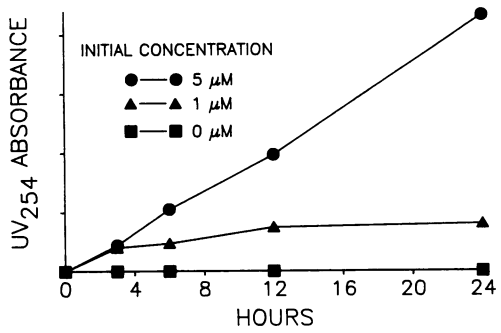

FIG. 3. Time course of accumulation of a UV 254-nm-absorbing material that comigrates with DOC on HPLC analysis. COS-1 cells were transfected with plasmid encoding P450IIC5. Incubation medium was supplemented with 0, 1, or 5 μM radio-inert 21-hydroxypregnenolone, as indicated.

17α-Hydroxypregnenolone is also metabolized by the rabbit liver 21-hydroxylase in a similar manner. The resultant products were identified as 17α,21-dihydroxypregnenolone and the 3-keto,Δ^4-analogous steroids of the above product and substrate (Fig. 5). Inhibition of this reaction with 10 μM clotrimazole was complete, whereas trilostane (2.5 μg/ml) reduced the rate of metabolism to half that of control (data not shown). As noted previously, trilostane may be able to inhibit reactions with K_m values higher than pregnenolone.

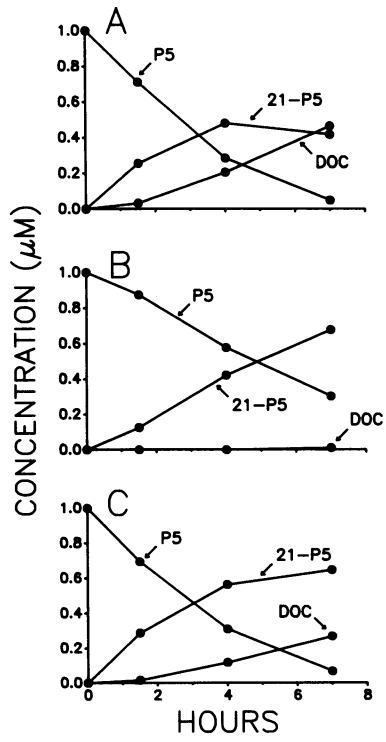

FIG. 4. Time course of tritiated pregnenolone (1 μM) metabolism by COS-1 cells transfected with a plasmid encoding P450IIC5. P5, pregnenolone; 21-P5, 21-hydroxypregnenolone. (*A*) Control. (*B*) Same as *A*, except supplemented with 10 μM clotrimazole. (*C*) Same as *A*, except supplemented with 2.5 μg of trilostane per ml. Mock-transfected COS-1 cells demonstrated no activity with pregnenolone, 17α-hydroxypregnenolone, or DHEA.

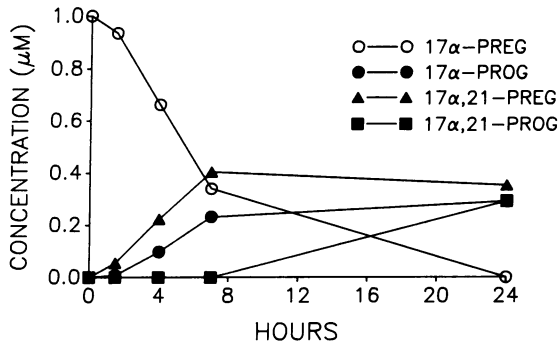

FIG. 5. Time course of tritiated 17α-hydroxypregnenolone (1 μM) metabolism by COS-1 cells transfected with plasmid encoding P450IIC5. 17α-Preg, 17α-hydroxypregnenolone; 17α-Prog, 17α-hydroxyprogesterone; 17α,21-Preg, 17α,21-dihydroxypregnenolone (3β,17α,21-trihydroxy-5-pregnene-20-one); 17α,21-Prog, 17α,21-dihydroxyprogesterone (11-deoxycortisol; 17α,21-dihydroxy-4-pregnene-3,20-dione).

The steroid 3β-hydroxy-5,16-pregnadiene-20-one (Δ^{16}-pregnenolone) is a UV 254-nm absorbing steroid: this property permits a facile monitoring of its metabolism. It was initially metabolized by COS cells expressing P450IIC5 to a steroid that coeluted with 3β,21-dihydroxy-5,16-pregnadiene-20-one, which was subsequently metabolized to 21-hydroxy-4,16-pregnadiene-3,20-dione.

Metabolism of C_{19} Androgens by Rabbit Liver 21-Hydroxylase. Although androgens lack the side chain containing C-21, DHEA is metabolized rather rapidly by rabbit liver 21-hydroxylase (Table 1). DHEA (1 μM) was rapidly metabolized to three products (Fig. 6). The major product was isolated and identified as 3β,16α-dihydroxy-5-androstene-17-one (16α-DHEA) by GC/MS. The other two polar metabolites remain unidentified. To identify these metabolites, media from incubations with 5 μM tritiated DHEA were collected, and the steroids were extracted. The residue was subjected to HPLC, and the fractions were collected. Fractions containing the polar steroids were found to contain 16-oxygenated DHEA (16α-DHEA, 3β,17β-dihydroxy-5-androstene-16-one, 16β-DHEA, and trace amounts of 3β,16α,17β-trihydroxy-5-androstene), a small amount of 3β,4α-dihydroxy-5-androstene-17-one (4α-DHEA), and 3β,7α-dihydroxy-5-androstene-17-one (7α-DHEA) as identified by GC/MS and listed above in descending order of abundance. Androstenedione was also isolated and identified by TLC. As seen with incubations with pregnenolone, 3β-HSD-like conversion of the substrate was evident only at high substrate concentrations (5 and 10 μM).

The rate of androstenedione metabolism was significantly slower than DHEA metabolism (Table 1). However, like DHEA, the major product of androstenedione metabolism was a 16α-hydroxylated substrate (data not shown). Trace amounts of other metabolites, which have retention times on either side of 16α-androstenedione, were detectable on HPLC analysis.

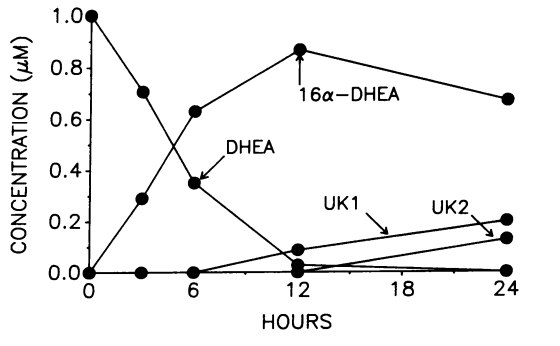

FIG. 6. Time course of tritiated dehydroepiandrosterone (1 μM) metabolism by COS-1 cells transfected with plasmid encoding P450IIC5. 16α-DHEA, 16α-hydroxyDHEA (3β,16α-dihydroxy-5-androstene-17-one); UK1 and UK2, unknown metabolites.

Mechanism of the 3β-HSD-Like Activity. To gain some insight into the mechanism of the 3β-HSD-like activity of COS cells expressing P450IIC5, the metabolism of three additional steroids was investigated. 3β-Hydroxy-4-pregnen-20-one (3β-progesterone) was converted to progesterone by both mock and transfected cells, but only the transfected cells converted it to DOC. The steroid did not spontaneously convert to progesterone in an aqueous environment. 3β,4α,17β-Trihydroxy-5-androstene, the steroid identified from incubations with DHEA, was not metabolized to a UV 254-nm-absorbing steroid by mock or transfected cells. 5-Androstene-3,17-dione was immediately epimerized (spontaneously) to androstenedione in an aqueous environment.

DISCUSSION

The hydroxysteroid dehydrogenase 17β-HSD is typically responsible for the interconversion of the functional group at the C-17 position of androgens and estrogens between hydroxyl and keto groups. It has been reported previously that a rat liver microsomal cytochrome P450-mediated reaction was capable of converting testosterone to androstenedione through the formation of a *gem*-diol at the C-17 position (27).

If a comparable mechanism is invoked for the reaction catalyzed by P450IIC5 at the C-3 position, then a 3-keto functional group can be generated. As demonstrated with 5-androstene-3,17-dione, a 3-keto,Δ^5-configuration is unstable and will immediately epimerize to a 3-keto,Δ^4-configuration in an aqueous environment. Thus, a likely mechanism for the 3β-HSD-like activity of P450IIC5 is through the formation of a *gem*-diol at the C-3 position, followed by dual hydrogen abstraction with water loss, and spontaneous epimerization (Fig. 7) (27). This contrasts with the classical, non-P450 oxidation of the 3β-hydroxy,Δ^5 configuration to the 3-keto,Δ^5 configuration (followed by spontaneous isomerization to the Δ^4 configuration), by 3β-hydroxysteroid dehydrogenase/$\Delta^{5\rightarrow4}$-isomerase. The protein encoded by both the human placental (28) and rat testis (M.C.L., J.M.T., and J.I.M., unpublished work) cDNAs, upon transfection into COS-1 cells, catalyzes conversion of the three major Δ^5-3β-hydroxysteroid substrates pregnenolone, 17α-hydroxypregnenolone, and DHEA to their Δ^4-3-ketosteroid counterparts.

There are a large number of reports of extraadrenal production of DOC (refs. 29 and 30, for example) and alternate pathways of DOC production through pregnenolone (29, 31). The significance of these pathways has not been fully established. However, it is interesting to note that most hepatic metabolism of steroids is catabolic. The reaction described here for P450IIC5 converts one functional hormone (progesterone) to

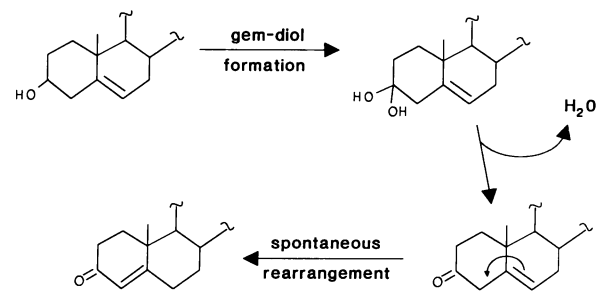

FIG. 7. Postulated mechanism of conversion of Δ^5-3β-hydroxysteroids to Δ^4-3-ketosteroids by formation of a *gem*-diol intermediate.

another (DOC). Factors that affect the relative occurrence of the cytochrome P450 isozymes may affect the metabolic fate of progesterone in much the same manner as they affect the balance between activation and detoxification of environmental carcinogens and toxins. The homologue to rabbit liver 21-hydroxylase has not been purified from human liver, but a number of animal/organ models have demonstrated similar activity: bovine liver (30), bovine adrenals (31), guinea pig spleen (29), rat adrenals (32, 33), and human adrenals (33). Further, this enzyme seems functionally polymorphic in New Zealand White rabbits (21, 34, 35), an observation that has not been factored into the potential physiological significance of this enzyme's contribution to steroid hormone homeostasis.

Our results show that P450IIC5 can metabolize a number of steroids in a variety of ways. Previously, only the 21-hydroxylation of progesterone by the purified protein had been rigorously studied (2). Although there is only 30% amino acid identity between hepatic 21-hydroxylase and bovine adrenal 21-hydroxylase, the rates of progesterone metabolism by the two enzymes are nearly identical. However, more recently, the 21-hydroxylation of pregnenolone and the 2-hydroxylation of estradiol-17β by hepatic cytochrome P450 has been reported (17, 18). The bovine adrenal enzyme primarily uses progesterone and 17α-hydroxyprogesterone as substrate (16). This report demonstrates that rabbit liver P450IIC5 catalyzes the metabolism of pregnenolone and 17α-hydroxypregnenolone, whereas 17α-hydroxyprogesterone is a very poor substrate.

In our hands, 2-hydroxyestrogens were not detected in experiments done with estradiol-17β and incubated with COS-1 cells expressing P450IIC5. It is likely that these unstable catechol estrogens are rapidly destroyed by COS cells or the purification procedure because no protection agent was added (18); however, a significant loss of estradiol-17β with time was not detected.

C_{19} steroids, specifically DHEA and androstenedione, were primarily 16α-hydroxylated. However, GC/MS identified a number of additional 16-oxygenated androgens as well as 4α- and 7α-hydroxylated androgens. Although purity of the substrate (DHEA) was not verified by GC/MS, various sites of attack of the steroid nucleus are probably possible considering the variety of substrates that this enzyme can use. As with pregnenolone, DHEA was converted to the 3-keto,Δ4 configuration only at high substrate concentrations (5 and 10 μM). The demonstration that androgens are 16α-hydroxylated by this enzyme provides further evidence for the role of a *gem*-diol formed at the C-3 position. Molecular modeling suggests that the C-16 and the C-3 carbons could be positioned similarly depending on whether the A or D ring initially enters the active site.

This work was supported in part by grants from the U.S. Public Health Service (NIGMS 16488, NIGMS 37942, NIGMS 31001, CA 30253, DK 34400, and 5-T32HD07190).

1. Black, S. D. & Coon, M. J. (1987) *Adv. Enzymol.* **60,** 35–87.
2. Dieter, H. H. & Johnson, E. F. (1982) *J. Biol. Chem.* **257,** 9315–9323.
3. Imai, Y. & Sato, R. (1974) *Biochem. Biophys. Res. Commun.* **60,** 8–14.
4. van der Hoeven, T. A., Haugen, D. A. & Coon, M. J. (1974) *Biochem. Biophys. Res. Commun.* **60,** 569–575.
5. Guengerich, F. P. (1977) *Mol. Pharmacol.* **13,** 911–923.
6. Wolf, C. R., Szutowski, M. M., Ball, L. M. & Philpot, R. M. (1978) *Chem. Biol. Interact.* **21,** 29–43.
7. Norman, R. L., Johnson, E. F. & Muller-Eberhard, U. (1978) *J. Biol. Chem.* **253,** 8640–8647.
8. Liem, H., Muller-Eberhard, U. & Johnson, E. F. (1980) *Mol. Pharmacol.* **18,** 565–570.
9. Johnson, E. F. (1980) *J. Biol. Chem.* **255,** 304–309.
10. Koop, D. R. & Coon, M. J. (1979) *Biochem. Biophys. Res. Commun.* **91,** 1075–1081.
11. Ingelman-Sundberg, M., Johansson, I. & Hansson, A. (1979) *Acta Biol. Med. Ger.* **38,** 379–388.
12. Miki, N., Sagiyama, T. & Yamano, T. (1980) *J. Biochem. (Tokyo)* **88,** 307–316.
13. Dieter, H. H., Muller-Eberhard, U. & Johnson, E. F. (1982) *Biochem. Biophys. Res. Commun.* **105,** 515–520.
14. Nebert, D. W., Nelson, D. R., Adesnik, M., Coon, M. J., Estabrook, R. W., Gonzalez, F. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E. F., Kemper, B., Levin, W., Phillips, I. R., Sato, R. & Waterman, M. R. (1989) *DNA* **8,** 1–13.
15. Johnson, E. F. & Griffin, K. J. (1985) *Arch. Biochem. Biophys.* **237,** 55–64.
16. Lorence, M. C., Trant, J. M., Mason, J. I., Bhasker, C. R., Fujii-Kuriyama, Y., Estabrook, R. W. & Waterman, M. R. (1989) *Arch. Biochem. Biophys.* **273,** 79–88.
17. Johnson, E. F., Schwab, G. E., Singh, J. & Vickery, L. E. (1986) *J. Biol. Chem.* **261,** 10204–10209.
18. Schwab, G. E. & Johnson, E. F. (1985) *Arch. Biochem. Biophys.* **237,** 17–26.
19. Raucy, J. L. & Johnson, E. F. (1985) *Mol. Pharmacol.* **27,** 296–301.
20. McManus, M. E., Minchin, R. F., Sanderson, N., Swartz, D., Johnson, E. F. & Thorgeirsson, S. S. (1984) *Carcinogenesis (London)* **5,** 1717–1723.
21. Tukey, R. H., Okino, S., Barnes, H. J., Griffin, K. J. & Johnson, E. F. (1985) *J. Biol. Chem.* **260,** 13347–13354.
22. Kronbach, T., Larabee, T. M. & Johnson, E. F. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 8262–8265.
23. Zuber, M. X., Simpson, E. R. & Waterman, M. R. (1986) *Science* **234,** 1258–1261.
24. Andersson, S., Bishop, R. W. & Russell, D. W. (1989) *J. Biol. Chem.* **264,** 16249–16255.
25. Mason, J. I., Murry, B. A., Olcott, M. & Sheets, J. J. (1985) *Biochem. Pharmacol.* **34,** 1087–1092.
26. Sheets, J. J., Mason, J. I., Wise, C. A. & Estabrook, R. W. (1986) *Biochem. Pharmacol.* **35,** 478–491.
27. Wood, A. W., Swinney, D. C., Thomas, P. E., Ryan, D. E., Hall, P. F., Levin, W. & Garland, W. A. (1988) *J. Biol. Chem.* **263,** 17322–17332.
28. Lorence, M. C., Murry, B. A., Trant, J. M. & Mason, J. I. (1989) *Endocrinology* **126,** 2493–2498.
29. Winkel, C. A., Wade, C. E., Danley, D. L., MacDonald, P. C. & Casey, M. L. (1983) *J. Steroid Biochem.* **19,** 1635–1638.
30. Ichikawa, Y., Hiwatashi, A. & Tsubaki, M. (1984) *FEBS Lett.* **167,** 131–136.
31. Franklin, S. O., Lieberman, S. & Greenfield, N. J. (1987) *J. Steroid Biochem.* **28,** 749–757.
32. Diedrichsen, G., Sinterhauf, K., Wolff, H. P. & Lommer, D. (1977) *J. Steroid Biochem.* **8,** 631–641.
33. Kaufmann, S. H. E., Sinterhauf, K. & Lommer, D. (1980) *J. Steroid Biochem.* **13,** 101–103.
34. Reubi, I., Griffin, K. J., Raucy, J. L. & Johnson, E. F. (1984) *J. Biol. Chem.* **259,** 5887–5892.
35. Johnson, E. F., Finlayson, M., Hujsak, C. M., Pendurthi, U. R. & Tukey, R. H. (1989) *Arch. Biochem. Biophys.* **273,** 273–280.