Mechanistic Kinship between Hydroxylation and Desaturation Reactions: Acyl−Carbon Bond Cleavage Promoted by Pig and Human CYP17 (P-450$_{17\alpha}$; 17α-Hydroxylase-17,20-lyase)†

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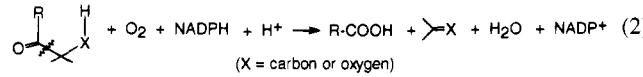
ABSTRACT: Using homogeneous pig and recombinant human CYP17, the mechanism of the acyl−carbon bond fission involved in the direct cleavage of pregnenolone was studied. It was found that the formation of androsta-5,16-dien-3β-ol (5,16-diene) and androst-5-ene-3β,17α-diol (17α-hydroxyandrogen) from pregnenolone was catalyzed by both the isoforms and that the two conversions were dependent on the presence of cytochrome b_5 (cyt b_5). 3β-Hydroxyandrost-5-ene-17β-carbaldehyde (aldehyde), an analogue of the physiological substrate pregnenolone, was handled as a substrate by both isoforms of CYP17. The aldehyde underwent cleavage to produce the 5,16-diene plus the 17α-hydroxyandrogen, at rates approximately 8- and 3-fold higher than any physiological reaction catalyzed, in the absence of cytochrome b_5, by the pig and human CYP17 isoforms, respectively. The stereochemistry of the reaction was studied using the aldehyde labeled with ^2H at three strategic positions, 16α, 16β, and 17α, with incubations performed under both $^{16}O_2$ and $^{18}O_2$. The results showed that the formation of the 5,16-diene is attended by the removal of the 16α-hydrogen atom; all three ^2H atoms are retained in the formation of 17α-hydroxyandrogen and its 17α-hydroxyl oxygen originates from O_2. Irrespective of the nature of the substrate, or the enzymic conditions used, the 5,16-diene and 17α-hydroxyandrogen were produced in similar ratios, suggesting that their genesis is closely linked. Both the compounds may be envisaged to arise from a peroxy adduct that fragments to give a carbon radical that then undergoes either a disproportionation or an oxygen-rebound reaction. The conclusion was supported by isotope-partitioning experiments when the conversion of a mixture of the unlabeled aldehyde and its isotopomer, containing ^2H at 16α as well as 16β, led to the enrichment of ^2H in 17α-hydroxyandrogen. It is suggested that the mechanistic kinship between hydroxylation and olefin formation, revealed by the present study, also applies to conventional hydroxylation and desaturation reactions.

Cytochrome P-450s constitute a large group of enzymes and contain a conserved cysteine, the thiolate group of which is ligated to the haem iron. Historically, these enzymes have been associated with hydroxylation reactions which occur according to the stoichiometry of eq 1 (Ortiz de Montellano, 1986; McMurry & Groves, 1986; Akhtar & Wright, 1991; Coon et al., 1992).

$$RH + NADPH + O_2 + H^+ \rightarrow ROH + NADP^+ + H_2O \quad (1)$$

Our work, directed initially to the elucidation of the mechanism through which certain methyl groups are removed during the biosynthesis of sterols and steroid hormones, then highlighted that two of the P-450 enzymes involved in the process, aromatase (Wright & Akhtar, 1990; Akhtar et al., 1982, 1993; Stevenson et al., 1985, 1988) and 14α-demethylase (Akhtar et al., 1978), catalyze not only the conventional hydroxylation reaction but also the oxidation of an alcohol into a carbonyl compound and more importantly an acyl−carbon bond cleavage reaction represented by eq 2.



(2)

An analogous situation was later envisaged for CYP17 (17α-hydroxylase-17,20-lyase, also P-450$_{17\alpha}$) which, in addition to the hydroxylation reaction, also catalyzes different types of side chain cleavages (Nakajin et al., 1981, 1985) (Scheme 1), all of which could be described broadly by the acyl−carbon bond cleavage of eq 2 (Stevenson et al., 1988; Akhtar et al., 1994a). The ability of these enzymes to catalyze different generic reactions at the same active site has been rationalized in terms of the model of Scheme 2 (Stevenson et al., 1988; Akhtar et al., 1994a). The main assumption underpinning the model is that, in the catalytic cycle of P-450s, the $Fe^{III}OOH$ species (**7**) is generated for conversion into the oxo derivative (**8**) that promotes hydroxylation by a free radical mechanism (Groves et al., 1978; Gelb et al., 1982; White et al., 1986; Atkinson & Ingold, 1993). However, when the target carbon atom of the substrate contains an electrophilic functionality, the iron peroxide is trapped, producing an adduct (**9**) which may

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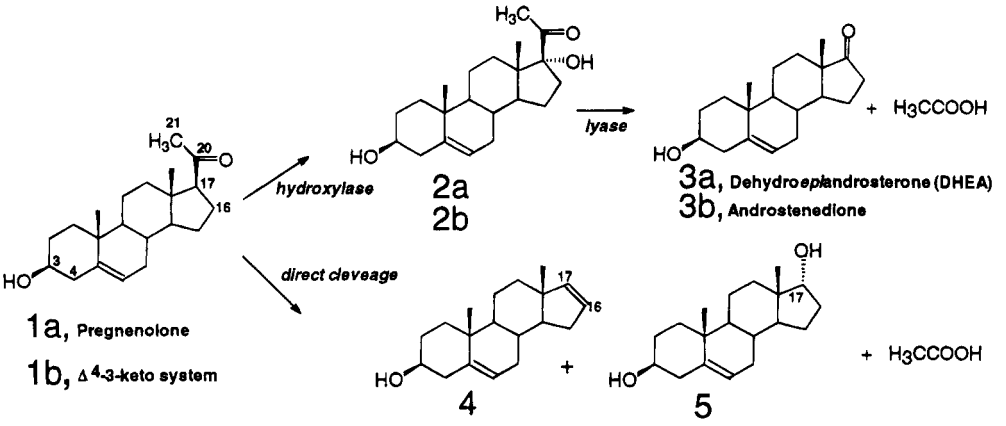
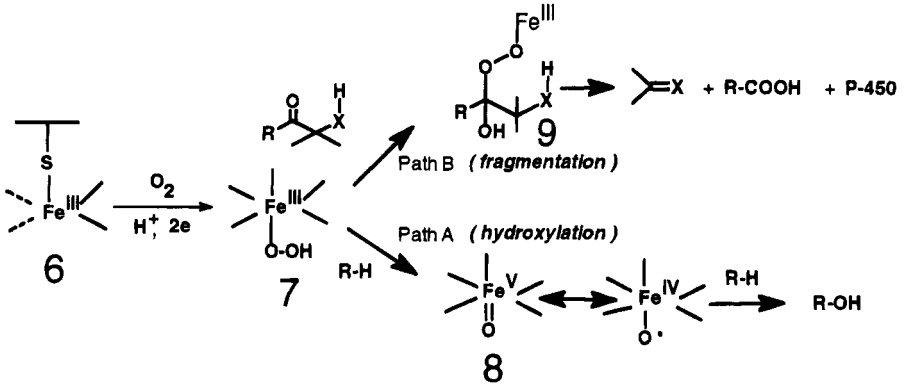
Scheme 1: Reaction Catalyzed by CYP17 Using the Three Activities (Hydroxylase, Lyase, and Direct Cleavage)



Scheme 2: $Fe^{III}OOH$ Species at the Crossroad of Hydroxylation (Path A) and Acyl−Carbon Bond Cleavage (Path B)



decompose by one of several closely related pathways, to furnish the fragmentation products (Akhtar & Wright, 1991; Akhtar et al., 1993). Of the hormonal multifunctional enzymes, CYP17 displays the greatest degree of promiscuity. Using its hydroxylase activity, CYP17 promotes the formation, from pregnenolone and progesterone, of the corresponding 17α-hydroxyprogestogens (**1** → **2**) which then, through its lyase activity, are converted into dehydroepiandrosterone (DHEA) (Nakajin et al., 1981) and androstenedione, respectively (**2** → **3**). In addition, the pig and human enzymes have been shown to cleave directly the pregnenolone side chain to produce the 5,16-diene (**4**) (Nakajin et al., 1985; Lee-Robichaud et al., 1995). Further, it is unambiguously shown here for the first time that both the isoforms of CYP17 are also responsible for the formation of 17α-hydroxyandrogen (**5**), which is conjectured to be the precursor of epitestosterone (Dehennin, 1993). This activity of CYP17, leading to the formation of **4** and **5**, is hereafter refered to as the direct cleavage activity.

In this paper, using the pig and human isoforms of CYP17, we provide experimental evidence for the involvement of a peroxide adduct of the type **9**, formed from the $Fe^{III}OOH$ species, in the direct cleavage process and also show that the adduct decomposes by a stepwise process which is best rationalized by invoking the participation of radical intermediates. Part of this work has been published in a preliminary communication (Robichaud et al., 1994).

EXPERIMENTAL PROCEDURES

[7-^{3}H]Pregnenolone was obtained from DuPont (U.K.) Ltd.; Optiphase Hisafe 3 from Wallac (U.K.); Synperonic NP10 ("Renex 690") from ICI Speciality Chemicals, Leatherhead (U.K.); Ni^{2+}-NTA-agarose from QIAGEN Inc., Chatsworth (U.S.A.); hydroxylapatite Bio Gel HTP from Bio-Rad Laboratories Ltd., Watford (U.K.); silica gel PF_{254} from Merck Darmstadt, Germany; NaB^3H_4 from Amersham Int., Amersham (U.K.); NaB^2H_4 from Aldrich Chemical Co., Gillingham (U.K.); $^{18}O_2$ gas (97%)−Ar gas mixture (1:2 ratio) from Isogas Ltd., Croydon, Surrey; and $^2H_2$ (99.5 atom % $^2H_2$) from Cambrian Gases, Croydon, Surrey. All other chemicals were obtained from Sigma Chemical Co. (U.K.). The plasmid pCW17mod, containing the modified human CYP17 gene, was a generous gift from Prof. M. R. Waterman, Vanderbilt University, Nashville, TN.

Chemical Synthesis of Isotopically Labeled Steroid Substrates

[21-^{3}H]-17α-Hydroxypregnenolone and [16α-^{3}H]pregnenolone were prepared using the general methods developed for the preparation of the deuteriated compounds (Akhtar et al., 1994a,b), and [17α-^{3}H]pregnenolone was prepared from 17α-bromopregnenolone following debromination with Zn in CH_3COO^3H (Kremers et al., 1974).

*3β-Hydroxyandrost-5-ene-17β-carbaldehyde (Aldehyde **10** Scheme 3).* 3β-Acetoxy-17-cyanoandrosta-5,16 -diene (0.6 g) (Butenandt & Schmidt-Thome, 1938, 1939) in tetrahydrofuran (20 mL) was hydrogenated using 5% palladium on barium sulfate (550 mg). After the uptake of hydrogen had been ceased, the solution was filtered and the solvent was removed *in vacuo*. The residue was subjected to silica gel column chromatography (3 × 54 cm), and the column was washed with petroleum ether (boiling point, 60−80 °C)

containing increasing amounts of ethyl acetate (up to 90%) to give 3β-acetoxy-17β-cyanoandrost-5-ene (0.47 g). This compound (200 mg), in tetrahydrofuran (10 mL), was treated at 0 °C with diisobutylaluminium hydride in toluene (3.6 mL, 1.5 M) and the mixture allowed to stand at room temperature for 30 min before 10% aqueous HCl (10 mL) was added. The mixture was then extracted with methylene chloride (2 × 15 mL), washed with water (2 × 10 mL), and dried with anhydrous sodium sulfate, and the solvent was evaporated *in vacuo* and the residue applied to a silica gel column (2 × 22 cm). 3β-Hydroxyandrost-5-ene-17β-carbaldehyde (80 mg) was eluted with petroleum ether−ethyl acetate (8:2) and had a mp of 150−155 °C [mp 148−153 °C (Miescher et al., 1940)]. Hitherto unreported data were as follows: *m/z* (relative intensity) 300 (7), 301 (5), 302 (100), 303 (22), 304 (5); ν_{max} (Nujol) 3540, 1725, 1705 cm^{-1}; δ_H ($CDCl_3$, 60 MHz) 0.77 (3H, s, 18-Me), 1.02 (3H, s, 19-Me), 3.5 (1H, br m, 3α-H), 5.35 (1H, m, 6-CH vinylic), 9.79 (1H, d, J = 1.6 Hz, 20-H).

*[16β-^{2}H]-3β-Hydroxyandrost-5-ene-17β-carbaldehyde (**10a**).* This compound was prepared as described immediately above but by using [16-^{2}H]-3β-acetoxy-17-cyanoandrosta-5,16-diene which in turn was obtained from [16α,16β-$^2H_2$]-3β-hydroxyandrost-5-ene-17-one and had, after correction for ^{13}C natural abundance, *m/z* (relative intensity) 303 (100) and 305 (6).

*[16α,16β,17α-$^2H_3$]-3β-Hydroxyandrost-5-ene-17β-carbaldehyde (**10b**).* The trideuteriated aldehyde was prepared from the [16-^{2}H]-3β-acetoxy-17-cyanoandrosta-5,16-diene as described above by substituting $^2H_2$ for H_2 in the hydrogenation step and had, after correction for ^{13}C natural abundance, *m/z* (relative intensity) 302 (2), 303 (1), 304 (28), and 305 (100).

*[16α,16β-$^2H_2$]-3β-Hydroxyandrost-5-ene-17β-carbaldehyde (**10c**).* This compound was prepared from the trideuteriated aldehyde (**10b**) by exchange with methanolic potassium hydroxide solution (4% w/v) for 3 days and had, after correction for ^{13}C natural abundance, *m/z* (relative intensity) 303 (8) and 304 (100).

*[16α,17α-$^2H_2$]-3β-Hydroxyandrost-5-ene-17β-carbaldehyde (**10d**).* This compound was prepared as described for **10**, except that $^2H_2$ was substituted for H_2 in the hydrogenation step, and had, after correction for ^{13}C natural abundance, *m/z* (relative intensity) 302 (5), 303 (27), 304 (100), and 306 (2).

Synthesis of [20-^{2}H]- or [20-^{3}H]-3β-Hydroxyandrost-5-ene-17-carbaldehyde and 3β-Hydroxyandrost-5-ene-17β-carbaldehyde. 21-Acetoxy-3β-hydroxypregn-5-en-20-one (100 mg) was dissolved in methanol (5 mL), NaB^2H_4 (50 mg) or NaB^3H_4 (0.1 mg, 30 mCi) was added, and the solution was stirred for 30 minutes. In the case of the NaB^3H_4 reduction, $NaBH_4$ (50 mg) was then added and the reaction continued for another 30 min. The solution was then made basic by the addition of a few drops of methanolic potassium hydroxide solution (5% w/v) and left at room temperature overnight. After neutralization with acetic acid, water was added to precipitate the product, which was filtered, washed with water, and dried *in vacuo*. This yielded 80 mg of pregn-5-ene-3β,20ξ,21-triol which was dissolved in methanol (6 mL) and treated with periodic acid (55 mg), which had been dissolved in methanol (1 mL) and neutralized with triethylamine. After 15 h at room temperature, the mixture was poured into water (50 mL) and extracted with ethyl acetate (2 × 50 mL). The combined organic layers were dried (Na_2SO_4) and evaporated under reduced pressure to give a white solid which was recrystallized from ethanol−water to give the desired product (50 mg) which was identical to authentic, unlabeled 3β-hydroxyandrost-5-ene-17β-carbaldehyde. The tritiated product had a specific activity of 7.5 × 10^4 dpm/nmol, and the deuteriated compound after correction for ^{13}C natural abundance gave *m/z* (relative intensity) 302 (1), 303 (100), and 304 (3).

Synthesis of [3α-^{3}H]-3β-Hydroxyandrost-5-ene-17β-carbaldehyde. 20ξ,21-(Isopropylidenedioxy)pregn-5-en-3β-ol (200 mg) (Steiger & Reichstein, 1938) was dissolved in dry dichloromethane (15 mL) and the mixture added to a suspension of pyridinium dichromate (220 mg) in dry dichloromethane (100 mL) and stirred at room temperature for 20 h. The chromium salts were then removed by filtration through Celite at a water pump. The filtrate was washed with water, dried (Na_2SO_4), and evaporated under reduced pressure to *ca.* 20 mL. Remaining brown color was removed by filtration through a small plug of silica gel (PF_{254}). The filtrate was again dried (Na_2SO_4) and the solvent removed under reduced pressure to give an oil which crystallized upon addition of light petroleum (bp 40−60 °C) to give 80 mg of crude 20ξ,21-(isopropylidenedioxy)pregn-5-en-3-one. As the sole contaminant was unoxidized starting material, the crude product was reduced with NaB^3H_4 without further purification as described for the synthesis of [20-^{3}H]-3β-hydroxyandrost-5-ene-17β-carbaldehyde (*vide supra*) to give crude [3ξ-^{3}H]-20ξ,21-(isopropylidenedioxy)pregn-5-en-3ξ-ol. This was deprotected by dissolving in methanol (5 mL) and treating with concentrated HCl (0.05 mL) at room temperature for 5 h. The volume of solvent was reduced (to *ca.* 2 mL) on a rotary evaporator, poured into water (50 mL), and extracted into ethyl acetate (2 × 30 mL). The combined organic layers were washed with aqueous sodium hydrogen carbonate and water, dried (Na_2SO_4), and evaporated under reduced pressure. The crude triol was purified on preparative TLC plates (silica gel PF_{254}) developed in ethyl acetate−acetone (4:1). Ethyl acetate−methanol (9:1) was used to elute the radioactive band (R_f = 0.51) corresponding to authentic pregn-5-ene-3β,20ξ,21-triol off the silica gel together with the contaminating 3α-hydroxy epimer (R_f = 0.54) which had not been completely resolved. After the solvent was removed and 100 mg of unlabeled triol was added, the mixture was cleaved with periodic acid, as described above for the synthesis of [20-^{3}H]-3β-hydroxyandrost-5-ene-17β-carbaldehyde. Chromatography on preparative TLC plates (silica gel PF_{254}), developed in dichloromethane−acetone (9:1), was used to separate the 3β-hydroxy epimer (R_f = 0.25) from the contaminating 3α-hydroxy epimer (R_f = 0.33). This gave the desired [3α-^{3}H]-3β-hydroxyandrost-5-ene-17β-carbaldehyde (18 mg) with a specific activity of 3.8 × 10^4 dpm/nmol.

Expression and Purification of Human CYP17

The expression, in *Escherichia coli*, of a modified form of human CYP17, using the plasmid pCW17mod, was performed by the method of Imai et al. (1993). The purification of the resulting enzyme, referred to as P-450H17mod(His)$_4$ by Imai et al. (1993), was performed as described therein but with the following modifications. (i) The detergent mixture of Synperonic NP13 (final concentration, 0.5% v/v) and Synperonic NP10 (final concentration, 0.5% v/v) was used instead of Emulgen 913 (final concentra-

tion, 1.0% v/v) and resulted in a 30% increased yield of P-450 solubilization. (ii) It was not necessary to filter the supernatant after solubilization and centrifugation. (iii) Stronger washing conditions were used for the Ni^{2+}-NTA-agarose column; a 20 mM histidine step was introduced after the glycine wash with the P-450 being eluted with 50 mM histidine. Finally, the two-column step used originally, to remove high-molecular weight protein impurities, substrate, and detergent, was condensed into a single column step as follows. P-450 was eluted off the Ni^{2+}-NTA-agarose column, dialyzed overnight, and then applied to a hydroxylapatite column (1 mL of bed volume/8 nmol of P-450), pre-equilibrated with sodium phosphate (20 mM) (pH 7.2), glycerol (20% v/v), progesterone (50 μM), and Synperonic NP10 (0.2% v/v). The column was washed with sodium phosphate (80 mM) (pH 7.2), glycerol (20% v/v), progesterone (50 μM), and Synperonic NP10 (0.06% v/v) (five column bed volumes) and then further with sodium phosphate (20 mM) (pH 7.2), glycerol (20% v/v), and EDTA (0.1 mM) (five column bed volumes). The P-450 was eluted with a step up to 300 mM sodium phosphate, and the colored fractions were pooled, dialyzed against sodium phosphate (20 mM) (pH 7.2), glycerol (20% v/v), and EDTA (0.1 mM), and stored at −70 °C. The resulting CYP17 gave a single band on Coomassie-stained 12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis. From 1.5 L of culture, 6.2 mg of homogeneous CYP17 was obtained, with a specific haem content of 7.04 nmol/mg of protein. Catalytic activities of this purified cytochrome, expressed as nanomoles of product formed per minute per nanomole of P-450, are listed in Table 2.

Purification of Porcine CYP17, Porcine NADPH−Cytochrome P-450 Reductase and Porcine Cytochrome b_5

The same batches of porcine 17α-hydroxylase-17,20-lyase (pig CYP17), NADPH−cytochrome P-450 reductase, and cytochrome b_5, which were purified (Suhara et al., 1984; Strobel & Dignam, 1978; Strittmatter et al., 1978) in a previous study (Lee-Robichaud et al., 1995), were also used in this work.

Enzyme Assays (Kinetic Data of Table 2)

Steroids (15 nmol) were incubated with CYP17 (30 pmol), NADPH−cytochrome P-450 reductase (1 unit), L-α-phosphatidylcholine dilauroyl (40 μg), and, when present, cytochrome b_5 (150 pmol), in a final volume of 1 mL, as described previously (Lee-Robichaud et al., 1995). The lyase activity (**2a** → **3a**, Scheme 1) was monitored by measurement of the release of tritiated acetate from [21−$^3H_3$]-17α-hydroxypregnenolone (specific radioactivity, 4.4 × 10^4 dpm/nmol) into the aqueous medium (Lee-Robichaud et al., 1995). [7-^{3}H]Pregnenolone (specific activity, 4.4 × 10^5 dpm/nmol) was used as substrate for measurement of the hydroxylase and direct cleavage activities by isolation of the labeled 17α-hydroxypregnenolone and androsta-5,16-dien-3β-ol, respectively (Lee-Robichaud et al., 1995). Cleavage of the aldehyde (**10**) was monitored by measurement of, at 0, 1, 2, 3, and 4 min, the release of tritiated formate into the medium from [20-^{3}H]aldehyde (0.25, 0.50, 1.0, 4.0, and 18.0 nmol; specific radioactivity, 7.5 × 10^4 dpm/nmol), using the same method as that described for the [21-$^3H_3$]-17α-hydroxypregnenolone substrate (Lee-Robichaud et al., 1995). The kinetic constants were derived from iterative nonlinear regression analysis of the primary velocity−time curves.

Preparative Scale Enzymic Incubations and Mass Spectrometric Analysis (Table 1, 3, and 4)

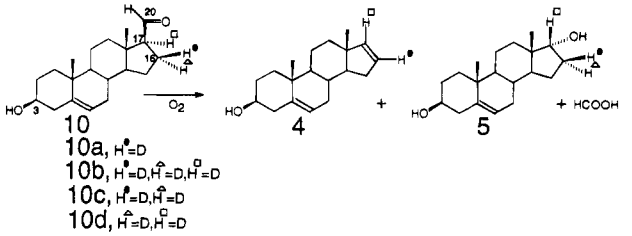
In order to obtain sufficient product for a range of analyses, the incubation was performed at 37 °C in sodium phosphate buffer (50 mM) (pH 7.2) and EDTA (0.1 mM) in a final volume of 3.5 mL. 3β-Hydroxyandrost-5-ene-17β-carbaldehyde (250 nmol), labeled variously with deuterium (16α, 16β, and 17α and their various combination), was admixed with unlabeled material (250 nmol) and a trace amount of 3-tritiated material (specific radioactivity, approximately 1 μCi). This substrate, in 15−20 μL of dimethylformamide, was mixed with L-α-phosphatidylcholine dilauroyl (400 μg), CYP17 (3.0 nmol), and NADPH−cytochrome P-450 reductase (4 units), made up to 1.5 mL with the buffer, and left to reconstitute for 3 h at 4 °C. The reconstituted mixture was preincubated at 37 °C for 5 min and the reaction started by the addition of preincubated NADPH-generating buffer (2.0 mL) [sodium phosphate (50 mM) (pH 7.2), EDTA (0.1 mM), $NADP^+$ (3 mM), glucose-6-phosphate (14 mM), and glucose-6-phosphate dehydrogenase (4 units)]. Incubations were terminated after 25 min by vortexing with 10 mL of ethyl acetate−methanol (9:1). The steroids were extracted in a further 2 × 10 mL of ethyl acetate−methanol (9:1) and the combined organic extracts washed with saturated brine, dried over anhydrous sodium sulfate. The solvent was evaporated down *in vacuo*, and the steroids were purified by silica gel TLC [developed twice in benzene−acetone (10:1), solvent front 20 cm]. The steroids were located by scanning for radioactivity, and following extraction of the silica [with ethyl acetate−methanol (9:1)], the bands containing androsta-5,16-dien-3β-ol (**4**) and androst-5-ene-3β,17α-diol (**5**) were combined and analyzed by GC−MS, either directly or following conversion to trimethylsilyl derivatives (Akhtar et al., 1994a). The latter procedure was used for incubations with human CYP17 when smaller amounts of products were formed, and the derivatization improved the sensitivity.

A mixture of known composition of the two steroids was also analyzed by SIR following the isotopically labeled determination, to obtain the ratios of the two steroid products (Table 3). For this purpose, the ratio of the intensities of the M^+ ions of each steroid in the reference mixture was shown to agree with the known mixture ratio. This allowed a calculation of the unknown ratio of the two steroid products, isolated together from the enzyme incubations, by merely calculating the sum of the intensities of the M^+ ions of all the isotopomers of a given steroid.

Incubations under $^{18}O_2$ Gas

The incubation is similar to the preparative scale protocol described above, with the following modifications. The vessel containing human CYP17, NADPH−cytochrome P-450 reductase, L-α-phosphatidylcholine dilauroyl, substrate, $NADP^+$, glucose-6-phosphate, and buffer underwent three rounds of deaeration under vacuum (water pump) and flushing with argon and was subsequently left at 37 °C for 10 min. Glucose-6-phosphate dehydrogenase (10 units) was added, followed immediately by two cycles of evacuation under reduced pressure and flushing with argon. After the

Scheme 3: Conversion of Various Isotopomers of the Aldehyde (**10**) into **4** and **5**[a]



[a] The fate and stereochemistry of the 16α-, 16β-, and 17α-hydrogen atoms, during the conversion, are shown by the appropriate symbols.

third evacuation, a 1:2 mixture of $^{18}O_2$ (99.5%) argon was introduced into the vessel and the tap closed. The incubation mixture was shaken at 37 °C for 1 h.

Analysis of ^{18}O Incorporation into the Released Formate and 17α-Hydroxyandrogen

[20-^{2}H]Aldehyde (200 nmol) was admixed with [16α,17α-^{2}H]aldehyde (250 nmol) and [20-^{3}H]aldehyde (50 nmol, 3.5 × 10^6 dpm) and incubated under $^{18}O_2$ gas as described above. Following acidification (10% aqueous phosphoric acid, 450 μL), the incubation was freeze-dried and the residue suspended in 4 mL of water, and the steroids were extracted, washed, dried, separated on silica TLC, and analyzed by GC−MS as described above. The distillate above was basified (to pH 7−8) with 35 μL of aqueous sodium hydroxide (0.4 M) and again freeze-dried. The residue after dissolution in water (3 × 200 μL), was transferred to a small vial and freeze-dried and the formate derivatized to the benzyl ester using the protocol previously described for acetate derivitization (Akhtar et al., 1994b). Benzyl formate was analyzed using the following GC−MS conditions: injector, 225 °C; initial column temperature, 70 °C for 4 min, programmed 4 °C/min to 120 °C for 1 min, and then 25 °C/min to 240 °C for 5 min, with sample transfer lines 240 °C. The retention time of the benzyl formate was 10.0 min. SIR traces were taken of the ions of mass 136.1−141.1 (M^+ = 136.1) and the full scans of range 150−80, resolution 500.

RESULTS

All the enzymes used in the present study were purified to homogeneity to give single bands on Coomassie-stained sodium dodecyl sulfate−polyacrylamide gel electrophoresis. The source of the human CYP17 was a plasmid (Imai et al., 1993) in which the 5′ end of the gene had been modified to allow its expression in *E. coli* and the 3′ end contained a tag of 12 nucleotides coding for four histidine residues, to facilitate the isolation of the gene product by chromatography, on a Ni^{2+} column. The enzyme was purified as described originally (Imai et al., 1993) but with the introduction of minor modifications throughout which shortened the procedure by one column step.

The versatility of CYP17 was then exploited to probe the behavior of the enzyme toward an analogue in which the ketone group of the substrate was replaced by a more electrophilic aldehyde functionality, as in structure **10**, Scheme 3. Attention is drawn to our previous results with the pig CYP17 (Lee-Robichaud et al., 1995) which confirmed that its hydroxylase and lyase activities were significantly stimulated by cytochrome b_5 (Suhara et al., 1984; Katagiri et al., 1982; Onoda & Hall, 1982) and that the direct cleavage activity, culminating in the formation of the 5,16-diene (**4**), was exclusively dependent on the presence of cytochrome b_5 (Nakajin et al., 1985; Meadus et al., 1993). The most noteworthy feature of the results with the human CYP17 that we (Lee-Robichaud et al., 1995) and others (Katagiri et al., 1995) had obtained is that 17α-hydroxypregnenolone (**2a**), formed by the hydroxylase activity, is converted into DHEA by its lyase activity, in significant quantity, only in the presence of cytochrome b_5. The requirement of cytochrome b_5 for the formation of the 5,16-diene by the direct cleavage activity is similar for the two isoforms (Lee-Robichaud et al., 1995). Table 1 presents results from pilot experiments in which the major metabolites produced by the two CYP17 isoenzymes, from the analogue aldehyde (**10**), were quantified. In these, the effect of cytochrome b_5 was also studied. The incubation of the analogue aldehyde (**10**) with either the pig or the human CYP17 led to its facile cleavage which was independent of cytochrome b_5.

*Kinetic Parameters Using the Physiological Substrates and the Analogue Aldehyde (**10**).* In view of these encouraging results, detailed kinetic parameters were obtained for the two isozymes, using the analogue aldehyde (**10**), pregnenolone, and 17α-hydroxypregnenolone as substrates (these determinations were performed in parallel to enable direct comparison). Table 2 shows that, in the case of the pig isoform, the V_{max} value for the cleavage of the analogue aldehyde (**10**) was over 8-fold higher than that of any other reaction catalyzed by the enzyme using the physiological substrates in the absence of cytochrome b_5 and over 3-fold higher than any in the presence of cytochrome b_5. The use of specificity constant K_{cat}/K_m for comparison reinforced the supremacy of the analogue aldehyde, giving its cleavage a distinct edge over the most favorable, cytochrome b_5-stimulated, physiologically important *lyase* activity, producing DHEA. A similar kinetic profile was shown by the human CYP17, though in this case, the results were less dramatic than with the pig isoform; the V_{max} values for the cleavage of the analogue aldehyde were only 3- and 1.3-fold higher than those recorded for the most favorable physiological reaction catalyzed by the enzyme in the absence and presence of cytochrome b_5, respectively, i.e. the hydroxylation of pregnenolone. This supremacy of the analogue aldehyde over the physiological substrate, pregnenolone, is further emphasized by comparing the direct cleavage activities; here, the analogue aldehyde is cleaved almost 15- and 7-fold higher than pregnenolone by the porcine and human CYP17 isoforms, respectively.

*Formation of the 17α-Hydroxyandrogen (**5**) Is the Property of CYP17.* We have recently performed the mechanistic analysis of five acyl−carbon bond fission reactions (Akhtar et al., 1994a). Of these, one each is catalyzed by aromatase and 14α-demethylase, two are catalyzed by CYP17, and the fifth is observed during the incubation of pregnenolone with pig testis microsome, resulting in the formation of the 17α-hydroxyandrogen (**5**). Detailed mechanisic studies on the conversion, pregnenolone (**1a**) → 17α-hydroxyandrogen (**5**), have led to the conclusion that in this case the acyl−carbon bond cleavage is best rationalized by involving the intermediacy of a peroxide adduct [Corina et al., 1991; Akhtar et al., 1994a; also see the earlier suggestion of Lynn and Brown (1958) for the conversion **2a** → **3a**]. Although it has been suspected that the cleavage **1a** → **5** is the property of CYP17,

Table 1: Product Profile from the Incubation of the Aldehyde Analogue (**10**) with Porcine, Bovine, and Human CYP17[a]

incubation condition	nanomoles of product/100 nmol of substrate incubated		
	17OH preg (**2a**)	DHEA (**3a**)	$\Delta^{5,16}$-diene (**4**)
aldehyde			
(1a) porcine CYP17	nd	nd	nd
(1b) porcine CYP17 + reductase	nd	4	75
(1c) porcine CYP17 + reductase + cyt b_5	nd	5	71
(2) bovine testes microsomes	nd	nd	37
(3a) human CYP17	nd	nd	nd
(3b) human CYP17 + reductase	nd	nd	35
(3c) human CYP17 + reductase + cyt b_5	nd	nd	30

[a] [3-^{3}H]Aldehyde (**10**) (500 nmol, specific radioactivity of 3.8×10^4 dpm/nmol) was incubated using the preparative scale protocol, and when present, 8 nmol of cytochrome b_5 was added. In experiment (2), aldehyde (200 nmol) was incubated with calf testes microsomes (400 mg of protein) in a fixed volume of 10 mL (phosphate buffer, pH 7.2). The steroid products were extracted and separated by silica gel TLC, as described in Experimental Procedures. The radiolabeled steroid products were quantified using a Berthold TLC scanner/counter. nd is not detectable.

Table 2: Kinetic Parameters of Purified Human and Porcine CYP17[a]

reaction	human				porcine			
	b_5	K_m (μM)	V_{max} mol min^{-1} P-450^{-1}	K_{cat}/K_m (M^{-1} s^{-1} $\times$ 10^{-3})	b_5	K_m (μM)	V_{max} mol min^{-1} P-450^{-1}	K_{cat}/K_m (M^{-1} s^{-1} $\times$ 10^{-3})
hydroxylase	−	0.68 ± 0.07	1.66 ± 0.07	41	−	0.33 ± 0.06	1.15 ± 0.15	58
(preg → 17OH-preg)	+	0.67 ± 0.06	3.61 ± 0.15	92	+	0.30 ± 0.04	2.00 ± 0.27	110
lyase	−	−	<0.15	−	−	0.29 ± 0.05	2.21 ± 0.21	130
(17OH-preg → DHEA)	+	0.62 ± 0.10	1.54 ± 0.12	41	+	0.30 ± 0.03	5.42 ± 0.52	300
direct cleavage	−	n/a	nd	n/a	−	n/a	<0.05	n/a
(preg → diene)	+	0.65 ± 0.06	0.69 ± 0.03	18	+	0.25 ± 0.08	1.26 ± 0.22	84
direct cleavage	−	0.85 ± 0.10	4.71 ± 0.97	92	−	0.76 ± 0.03	18.62 ± 0.44	410
(aldehyde → diene)	+	−	4.70[b]	−	+	−	18.6[b]	−

[a] Activity assays were performed as described in Experimental Procedures and the data processed by iterative nonlinear regression analysis of the primary velocity−time curves. [b] Substrate-saturating rate assays (data not shown) confirmed that cytochrome b_5 conferred no change on the rate of cleavage of the aldehyde and so kinetic studies were not performed, as indicated by dashes in the relevant row. nd is not detectable; n/a is not applicable.

Table 3: Ratios of Androsta-5,16-dien-3β-ol (**4**) and Androst-5-ene-3β,17α-diol (**5**), Produced by Human and Porcine CYP17, under Different Conditions[a]

substrate	cyt b_5	% of total radioactivity recovered		ratio of products **5**:**4**
		17α-hydroxyandrogen (**5**)	diene (**4**)	
human				
[16α,17α-^{2}H]pregnenolone[b]	−	nd	nd	0:0(2)
	+	1.7 ± 0.1	5.8 ± 0.4	1:3.4 ± 0.1(5)
[16β-^{2}H]aldehyde[c]	−	8.2 ± 1.2	32.0 ± 0.5	1:3.9 ± 0.7(2)
[16α,17α-^{2}H]aldehyde[c]	−	6.9 ± 1.3	20.7 ± 2.6	1:3.0 ± 0.4(4)
[16α,16β-^{2}H]aldehyde[c]	−	7.3 ± 0.8	24.0 ± 1.4	1:3.3 ± 0.3(4)
porcine				
[3α,20-^{2}H]aldehyde[b]	−	11.4 ± 1.6	36.0 ± 1.7	1:3.2 ± 0.6(6)

[a] Incubations were performed and steroids extracted and separated as described in Experimental Procedures. Figures represent the mean value ± standard deviation several independent incubations, the number of which are given in parentheses. [b] For these incubations, the ratios of steroid products were calculated from the ratio of radioactive peaks corresponding to the separated steroid, which were then later identified by GC−MS. [c] The ratios of steroid products were calculated using GC−MS as described in Experimental Procedures. nd is not detectable.

this fact had not been established unambiguously (Shimizu, 1978; Weusten et al., 1989). In our view, the preceding conversion occupies a central position in the study of the mechanism of the acyl−carbon bond fission. It is therefore important to fully characterize the enzyme involved in the process, and the availability of two highly purified isoforms of CYP17 has made the task possible.

The 17α-hydroxyandrogen (**5**), arising from the direct cleavage activity, will have two "birth marks"; the 17α-hydroxyl oxygen will originate from O_2, and all three hydrogen atoms at the two strategic positions of the precursor, 16α, 16β, and 17α, will be retained in the product. In view of this consideration, the search for the formation of such a species was made using the precursor labeled with ^{3}H as well as ^{2}H. The radioactivity of the former isotope served to quantify the conversion, while strategic placement of ^{2}H was used to aid the characterization of the product by mass spectrometry. Attention has already been drawn to the fact that the direct cleavage of the side chain of pregnenolone, by both the isozymes to give the 5,16-diene (**4**), is exclusively dependent on the presence of cytochrome b_5 (Lee-Robichaud et al., 1995). The data of Table 3 show the formation, from pregnenolone, of 17α-hydroxyandrogen (**5**), as well as the 5,16-diene(**4**), only in the presence of cytochrome b_5. The analysis of the metabolites produced by the two isozymes, from the analogue aldehyde (**10**), also showed the formation of the 17α-hydroxyandrogen. The ratios of the two direct cleavage products (17α-hydroxyandrogen and 5,16-diene) were the same for both the enzymes and in all the experiments recorded in Table 3. Given that quantification of 17α-

hydroxyandrogen (**5**) and 5,16-diene (**4**), in the present study, involves a large number of manipulations, each prone to its own experimental error, it is interesting to note that the average ratios for the two products are within a narrow range of 3−4 and very similar to the ratio obtained with the pig microsomes in our earlier study [see the legend to Table 1 in Akhtar et al., (1994a)]. That 17α-hydroxyandrogen (**5**) and 5,16-diene (**4**) are the products of the direct cleavage activity will be shown by a deuterium labeling approach in the next section, but the main message from the experiments described above is that, irrespective of the nature of the substrate or the isozyme used, the formations of the 5,16-diene (**4**) and the 17α-hydroxyandrogen (**5**) are closely linked processes.

*Stereochemical and Isotopic Studies on the Conversion of the Aldehyde (**10**) into **4** and **5** (Scheme 3).* The thrust of the next set of experiments was two-fold: to determine the substrate stereospecificity of the cleavage of the analogue aldehyde and also to examine the operation of any isotope effect during the two conversions **10** → **4** + **5**. The syntheses of the various deuteriated isotopomers of the aldehyde are described in Experimental Procedures; each of these was admixed with the unlabeled material to give an approximate 1:1 mixture. As will be seen, the use of a mixture of known composition provided an internal control so that the behavior of the deuteriated species could be related to its protio counterpart and the information used for quantification and the assessment of isotope effect. In the conversion of the [16β-^{2}H]aldehyde (**10a**) with either the pig or the human CYP17, the ratios of the protio to deuterio species in the two products were, within experimental error, the same as in the precursor (entries 1 and 4, Table 4). The results showing the retention of the 16β-deuterium in both the products, though expected from our previous studies on the stereospecificity of the CYP17-catalyzed conversion using the physiological substrate pregnenolone (Corina et al., 1991; Akhtar et al., 1994a), provided the all important assurance that the methodology used in the present work was quantitatively reliable. When a similar mixture containing [16α,16β,17α-^{2}H$_3$]aldehyde (**10b**) was used as the substrate, with the pig enzyme, the 5,16-diene contained around two-thirds (62%) of the original deuterium (entry 2). The result clearly shows the loss of one deuterium during the conversion, but which of the three deuterium atoms that is removed in the process is not revealed by this experiment. This issue was later clarified using the human enzyme. The most significant finding from the experiment (entry 2), however, was the suggestion of the enrichment of deuterium in the 17α-hydroxyandrogen, with two independent experiments showing the same trend. When the labeled component in the mixture contained deuterium atoms in the 16α- and 17α-positions (**10d**), the diene was found to contain 40−47% of the original deuterium (entries 3 and 6). These results corroborate those obtained with the trideuteriated precursor. Regarding the isotopic composition of the 17α-hydroxyandrogen, the mass spectrometric analysis showed that its deuterium:protium ratio was the same as in the precursor (entries 3 and 6).

In order to critically evaluate the results foreshadowed in entry 2, for the formation of the 5,16-diene (**4**) as well as the 17α-hydroxyandrogen (**5**) from the trideuteriated precursor, it was considered important to focus exclusively at C-16. For this purpose, the C-17α-deuterium of **10b** was removed

Table 4: Isotopic Composition of Steroids Produced by Porcine and human CYP17 with Variously Deuteriated 3β-Hydroxyandrost-5-ene-17β-carbaldehyde (**10**)[a]

substrate and % isotopomer in the sample	*n*	% distribution of each isotopomer										
		diene (**4**)					17α-hydroxyandrogen (**5**)					
		U	D_1	D_2	D_3	% retention of ^{2}H	U	D_1	D_2	D_3	% retention of ^{2}H
porcine CYP17											
(1) [16β-^{2}H]aldehyde (55% U, 45% D_1)	1	57	43	–	–	96	52	48	–	–	107
(2) [16α,16β,17α-^{2}H]aldehyde (44% U, 6% D_1, 16% D_2, 34% D_3)	2	51	12 ± 1.0	37 ± 1.0	–	62 ± 0.5	35.5 ± 2.5	4.5 ± 0.5	22 ± 2.0	38 ± 4.0	116 ± 6.0
(3) [16α,17α-^{2}H]aldehyde (45% U, 12% D_1, 43% D_2)	2	61 ± 4.0	39 ± 4.0	–	–	40 ± 4.0	44.5 ± 1.5	13.5 ± 1.5	42 ± 3.0	–	100 ± 4.0
human CYP17											
(4) [16β-^{2}H]aldehyde (48% U, 52% D_1)	2	45 ± 1.0	55 ± 1.0	–	–	106 ± 2.0	45.5 ± 0.5	54.5 ± 0.5	–	–	105 ± 1.0
(5) [16α,16β-^{2}H]aldehyde (47% U, 9% D_1, 44% D_2)	3	48 ± 3.6	52 ± 3.6	–	–	54 ± 3.5	38 ± 2.5	5.0 ± 1.3	57 ± 1.9	–	123 ± 4.2
(6) [16α,17α-^{2}H]aldehyde (47% U, 14% D_1, 39% D_2)	3	57 ± 2.2	43 ± 2.2	–	–	47 ± 2.6	48 ± 1.2	12 ± 0.8	40 ± 0.5	–	100 ± 1.7

[a] Each isotopomer of the aldehyde was admixed with the unlabeled material to give the composition shown in column 1, and following incubations, the GC−MS analysis was performed, as described in Experimental Procedures. The figures represent mean values ± standard deviation of *n* numbers of independent incubations. In calculating the percentage distribution, the contributions due to ^{13}C natural abundance have been subtracted from the appropriate peaks; hence, the values given are true percentages of the isotopomers. U denotes the unlabeled species, and D_1, D_2, and D_3 are one-, two-, and three-deuterium-containing species, respectively. Isotopomers present in less than 2% of the total are denoted by a dash.

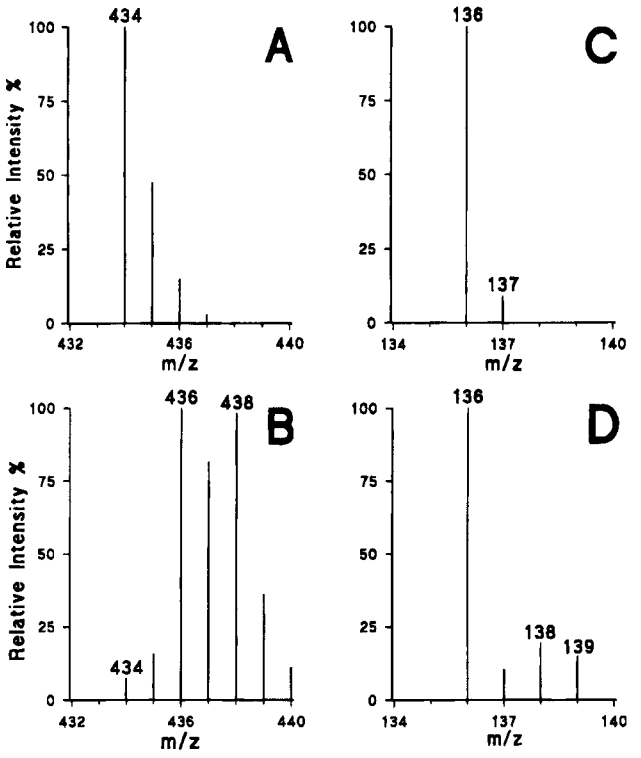


FIGURE 1: Incorporation of ^{18}O into androst-5-ene-3β,17α-diol (**5**) and formate. Incubations were performed using a mixture of the [20-^{2}H]aldehyde (**10**) (200 nmol), 16α,17α-dideuteriated species (**10d**) (250 nmol), and [20-^{3}H]aldehyde (50 nmol, SA 3.8 $\times$ 10^4 dpm/nmol) under ^{16}O or ^{18}O gas, as described in Experimental Procedures. The biosynthetic androst-5-ene-3β,17α-diol and cleaved formate side chain were worked up, converted to bis(trimethylsilyl) ether and benzyl formate, respectively, and subjected to GC–MS analysis as described in Experimental Procedures. (A) Unlabeled reference androst-5-ene-3β,17α-diol (M^+, 434), (B) androst-5-ene-3β,17α-diol isolated from ^{18}O incubations (*m/z* 436 and 438 for the protio and dideuterio species labeled with ^{18}O, respectively), (c) unlabeled reference formate (M^+, 136), and (D) formate isolated from ^{18}O incubations (*m/z* 136, 138, and 139 for the endogenous formate and the protio and deuterio formate labeled with one ^{18}O, respectively).

by exhaustive exchange and the resulting dideuteriated species (**10c**) mixed with the unlabeled material. The incubation of this mixture with human CYP17 clearly showed the loss of half of the ^{2}H (entry 5). This finding together with the data above established that, in the formation of the 5,16-diene (**4**), it is the 16α-hydrogen atom that is removed, precisely the same stereospecificity which has been deduced for the cytochrome b_5 dependent formation of the diene from the physiological substrate, pregnenolone (Kohara & Shimizu, 1987; Corina et al., 1991; Akhtar et al., 1994a). Once again, in all three experiments performed under entry 5, the mass spectrometric data showed the enrichment of deuteriated isotopomers in the 17α-hydroxyandrogen.

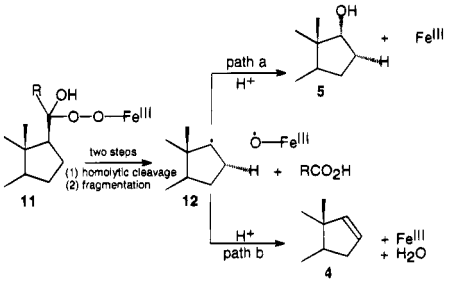
The findings detailed above are consistent with the notion that the formation of the 5,16-diene (**4**) and 17α-hydroxyandrogen (**5**) occur by a direct cleavage process, conforming to the requirement of eq 2. The outstanding issues remaining are the analysis of the C_1 fragment released during the process to determine the status of its oxygen atoms and also the evaluation of the origin of the 17α-hydroxyl oxygen of the 17α-hydroxyandrogen. The results pertaining to these aspects are presented in Figure 1 and show that the C_1 fragment is released as formate and one of its oxygen atoms is derived from molecular oxygen as is the hydroxyl oxygen of 17α-hydroxyandrogen.

DISCUSSION AND CONCLUSIONS

The study is concerned with the evaluation of the model of Scheme 2, which envisages that multicatalytic P-450s promote the hydroxylation and acyl–carbon bond cleavage reactions using two different types of iron–oxygen species. The Fe^{III}OOH species (**7**) formed from the resting state of P-450, O_2, and a hydride equivalent is normally directed to the formation of the oxo derivative (**8**) that participates in hydroxylation. However, when the sensitive carbon atom in the substrate contains a suitably juxtaposed electrophilic center, the Fe^{III}OOH species is trapped to produce the adduct (**9**), which decomposes to furnish the fragmentation products. The main goals of the present work were to provide experimental evidence for the involvement of the Fe^{III}OOH species in the process **7** → **9** and also evaluate the possible mechanism for the decomposition of the resulting adduct. For this purpose, we have exploited the relatively broad substrate specificity of CYP17 and have shown that the aldehyde (**10**), an analogue of pregnenolone, is handled by the human and pig isoforms of CYP17 as a substrate. More importantly, the pig enzyme metabolizes the analogue aldehyde about 8 times faster than any other reaction catalyzed by the enzyme, in the absence of cytochrome b_5 (Table 2). By and large, this trend is retained when the specificity constant, K_{cat}/K_m, is used as a yardstick for comparison. CYP17 displays three types of activities (hydroxylase, lyase, and direct cleavage), but with the analogue aldehyde (**10**), the hydroxylase activity is not used to any significant extent and it exclusively undergoes direct cleavage. The generic nature of the direct cleavage reaction and its exclusiveness and speed are fully consistent with the main requirement of the model in Scheme 2. The carbonyl group of the analogue aldehyde (**10**) is expected to be considerably more electrophilic than that of the physiological substrate and is able to provide a powerful trap for directing the highly nucleophilic Fe^{III}OOH species toward the fragmentation pathway. Quantitatively similar results are obtained with the human isoform, though in this case, the V_{max} and K_{cat}/K_m parameters for the cleavage of the aldehyde are only as good as the most favorable physiological reaction catalyzed by the enzyme, i.e. cytochrome b_5-stimulated hydroxylation (Table 2). In the case of the human CYP17, if the comparison is confined to the acyl–carbon cleaving reaction (i.e. lyase and direct cleavage activities), the V_{max} and K_{cat}/K_m values for these are higher for the analogue than for the related reactions involving the physiological substrate, in the presence or absence of cytochrome b_5.

The examples where designed compounds act as substrates for enzymes superior to their natural substrates are rare. That the aldehyde analogue should show such a behavior with two isoforms of CYP17 provides the chemical imperative in support of the involvement of a nucleophile such as Fe^{III}OOH (**7**), at a crucial stage in the overall enzyme process. Support for our original proposal that a peroxide may promote an acyl–carbon bond cleavage is provided by several other findings. These include studies on aromatase (Ranjith et al., 1993), aromatase model systems (Cole & Robinson, 1988; Oh & Robinson, 1993), and the use of artificial oxygen donors (Vaz et al., 1991; Roberts et al., 1991). Furthermore, during the reaction catalyzed by 14α-

Scheme 4: Mechanism of the Formation of **4** and **5** from the Peroxy Adduct (**11**)[a]



[a] The two-step sequence results in the release of RCO_2H as well as the formation of $Fe^{III}O^{\bullet}$ plus the carbon radical (**12**). The latter two species then produce **5** by an oxygen rebound reaction or **4** through disproportionation.

demethylase and CYP17, O-acyl derivatives have been isolated, the formation of which is best rationalized by invoking the Baeyer–Villiger rearrangement of the corresponding peroxide (Fischer et al., 1991; Mak & Swinney, 1992). It should, however, be emphasised that, in our original mechanistic analysis, the fact that peroxides of the type **9** may undergo Baeyer–Villiger rearrangement was considered, but it was found that such a reaction was not involved in oestrogen biosynthesis, catalyzed by aromatase (Akhtar et al., 1982, 1993). In keeping with the latter finding, we have now shown that 17-acetoxyandrost-5-en-3β-ol is not converted by CYP17 into the corresponding Δ^{16}-compound (data not shown). Thus, the isolation of O-acyl derivatives, though certainly telltale evidence that peroxides may be involved in the catalytic cycles of these multifunctional P-450s, does not reveal whether these compounds are merely side products or bona fide intermediates.

Next, the stereochemical analysis of the two conversions was performed, and it was established that, in the formation of the 5,16-diene (**4**) from the analogue aldehyde (**10**), it is the 16α-hydrogen of the substrate that is removed. The same steric course is displayed by both the CYP17 isoforms, which in turn is in complete consonance with the stereochemistry previously established for the formation of the diene (**4**) from the physiological substrate, pregnenolone (Kohara & Shimizu, 1987; Miller et al., 1991; Corina et al., 1991; Akhtar et al., 1994a). The 17α-hydroxyandrogen arising from the analogue aldehyde (**10**) retained all three hydrogen atoms, resident at C-16 and C-17, with its hydroxyl group originating from molecular oxygen. The cleavage of the C-17-C-20 bond of the substrate (**10**) and the formation of the new C–O bond in the product thus occur with the overall inversion of stereochemistry, as was previously shown for the genesis of the compound from pregnenolone using the pig testis microsomes (Shimizu, 1978; Miller et al., 1991; Corina et al., 1991; Akhtar et al., 1994a). The labeling data and stereochemical outcome forcefully emphasize that both the isoforms of CYP17 promote the formation of the 17α-hydroxyandrogen by a stepwise process. Although the nature of the intermediates participating in the conversion are not revealed by the experiments, chemical considerations dictate these to be free radical species with the overall conversion occuring by the sequence of Scheme 4.

The demonstration that the formation from pregnenolone and the analogue aldehyde (**10**) of the 17α-hydroxyandrogen (**5**) is the bona fide property of CYP17 and the serendipity that the structural changes involved in the process can be rationalized by a unique mechanism have a knock-on implication to the understanding of the mechanisms of other reactions catalyzed by the enzyme. The data in Table 2 and 3 clearly show that, for the direct cleavage of pregnenolone, by both isoforms, cytochrome b_5 is obligatorily required when the 5,16-diene (**4**) as well as 17α-hydroxyandrogen (**5**) is produced; in the absence of cytochrome b_5, neither of the two products is detected. For the cleavage of the analogue aldehyde (**10**), cytochrome b_5 is not required, and both the fragmentation products are coproduced. More significantly, irrespective of the nature of the substrate or the enzymic condition used, the ratio of the 5,16-diene to 17α-hydroxyandrogen is, within experimental error, very similar. In our view, these features provide strong circumstantial evidence to suggest that the formation of the two metabolites are closely coupled events. The mechanism considered above (Scheme 4) for the formation of the 17α-hydroxyandrogen also contains the essential elements required for rationalizing the formation of the diene (**4**). Both these steroids may be envisaged to arise by a common process in which the decomposition of the peroxy adduct (**11**) gives the carbon radical (**12**), which either furnishes the diene by a disproportionation reaction (path b, Scheme 4) or the 17α-hydroxyandrogen through oxygen rebound (path a). The two modes of neutralization of the carbon radical are in keeping with the behavior of such species in nonenzymic reactions. Another point to note is that in both cases the bonding events occur from the α-face of the steroid molecule, which indicates, but does not prove, that two products may arise from a common Michaelis complex. Support for the latter proposition is provided by the isotope data in Table 4.

As far as we are aware, isotope effects for hydrogen abstraction during disproportionation reactions of the type **12** → **4** (Scheme 4) have not been determined in nonenzymic systems, but on intuitive grounds, these are expected to be very small. Since the standard deviation in the experiments reported here is about $\pm 5\%$, we will not expect to observe small differences between the deuterium;protium ratios of the precursor versus the products. Notwithstanding this, the isotope distribution in Table 4 shows that, with both the isoforms of CYP17, when the labeled component in the substrate mixture contained two deuterium atoms at the same carbon atom, i.e. C-16, there was a significant enrichment of ^2H in the 17α-hydroxyandrogen (**5**) (entries 2 and 5, Table 4), presumably due to the operation of a reverse isotope effect. That, in the absence of a reliably detectable primary or secondary isotope effect, the sum of the two was clearly observable, though surprising, is gratifying. Irrespective of the theoretical implication of the results, the enrichment of ^2H in **5** provides the important pointer for the formation of it and the diene through the partitioning of a common carbon radical. The latter, because of the operation of a combined primary plus secondary isotope effect in hydrogen abstraction, during the disproportionation process, shows a small, but measurable, bias toward following the oxygen rebound route.

The preceding line of argument suggests a close kinship between hydroxylation and olefin-forming reactions, as was previously suggested for conventional hydroxylation and desaturation processes (Rettie et al., 1988; Akhtar & Wright, 1991) in which the crucial carbon radical is produced through the cleavage of a C–H rather than an acyl–carbon bond, as

studied here. The dividing line between the hydroxylation and desaturation is thus likely to be a fine one, and the enzymes which catalyze one or the other of these processes must have evolved with a particularly high degree of precision. In some cases, the precise organization within the Michaelis complex may be disturbed through the use of "wrong substrates" when otherwise monofunctional enzymes may be directed to produce olefinic as well as hydroxylated products, as has thus far been observed only in microsomal and whole cell systems (Rettie et al., 1988; Buist & Marecak, 1991).

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