Mechanism of the Acyl-Carbon Cleavage and Related Reactions Catalyzed by Multifunctional P-450s: Studies on Cytochrome P-450$_{17\alpha}$[†,‡]

Muhammad Akhtar,* David Corina, Sharon Miller,[§] Akbar Z. Shyadehi, and J. Neville Wright

Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton, England SO9 3TU

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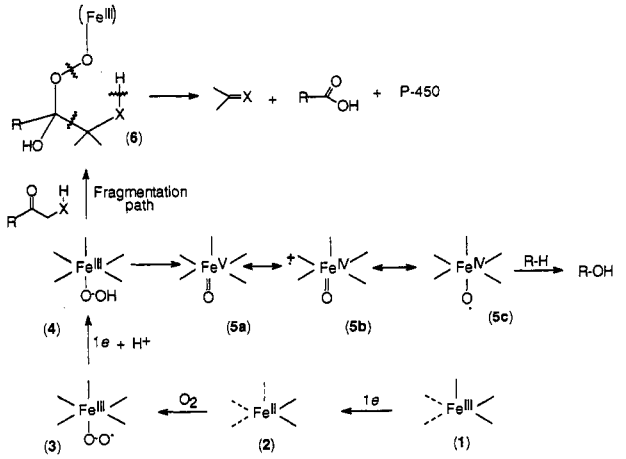
ABSTRACT: It is now well-known that conventional cytochrome P-450s catalyze hydroxylation reactions using an iron mono-oxygen species, the structure of which, as inferred from chemical model studies, may be represented by the following canonical forms: $Fe^{V}=O \leftrightarrow (\bullet+)Fe^{IV}=O \leftrightarrow Fe^{IV}—O^{\bullet}$. Certain multifunctional P-450s, notably those involved in steroid biosynthesis, catalyze, in addition to hydroxylation reactions, an acyl-carbon cleavage process in which the participation of an iron peroxide intermediate, Fe^{III}—OOH, has been suggested. However the possibility still exists that the C—C bond cleavage may also occur using the $Fe^{V}=O$ species. We have scrutinized the chemical consequences of involving either an $Fe^{V}=O$ or an Fe^{III}—OOH species for five different C—C bond cleavage reactions. With respect to the status as well as the origin of hydrogen and oxygen atoms, in four of the examples the mechanism involving the $Fe^{V}=O$ species makes the same prediction as that using the iron peroxide intermediate, that is, the incorporation of an atom of oxygen from O_2 into acyl part of the cleaved fragment. The fifth example, however, involving the formation, with pig testes microsomes, of 17α-hydroxyandrogen (androst-5-ene-3β,17α-diol) from pregnenolone, presents an interesting contrast—in this case different outcomes are predicted by the two mechanisms. These possibilities have been experimentally evaluated using substrates stereo- and regiospecifically labeled with heavy isotopes and incubated with pig testes microsomes under either $^{16}O_2$ or $^{18}O_2$. The first set of experiments established that, in the formation of the 17α-hydroxyandrogen from pregnenolone, the 16α, 16β, and 17α hydrogen atoms of the precursor are retained while an atom of oxygen from O_2 is incorporated into the steroid product. These findings require that if an $Fe^{V}=O$ species is involved in the crucial C—C bond cleavage process, then the initial abstraction of hydrogen must occur from C-21. This scenario necessitates that the side chain is released as ketene and converted into acetic acid by hydration. Consequently the acetic acid so produced should contain only two of the three hydrogen atoms from the C-21 methyl group and incorporate an atom of oxygen from H_2O. The results obtained in this work show that this is not the case and that all three hydrogen atoms from the C-21 methyl group are retained in acetic acid and one of its oxygens is derived from O_2. In the light of these observations and other findings, the biogenesis of the 17α-hydroxyandrogen is best rationalized by invoking the involvement of an Fe^{III}–OOH species.

It is now generally recognized that, in the hydroxylation reactions catalyzed by the P-450 group of enzymes, the activation of oxygen is achieved through the sequence of Scheme 1 [for reviews see McMurray and Groves (1986), Ortiz de Montellano (1986), Akhtar and Wright (1991), and Coon *et al.* (1992)]. The first step in the scheme is the binding of the substrate R–H to the resting Fe^{III} state of the enzyme, to give a binary complex (not shown) that undergoes a stepwise addition of two electrons and O_2 to produce an iron peroxide intermediate (**4**). Thereafter, the cleavage of the O–O bond in the peroxide furnishes an iron mono-oxygen species designated as the oxo derivative. From chemical model studies and by analogy with the structure of a related species produced during the course of the reaction catalyzed by horse radish peroxidase, the oxo derivative may be represented by the structure **5a**, in which the formal oxidation state of iron is Fe^{V}. The oxo derivatives of iron porphyrins however exist as radical cations (cf. **5b**) (Dolphin *et al.*, 1971; McMurray &

Scheme 1: Formation and Involvement of Various Iron–Oxygen Species in Cytochrome P-450-Catalyzed Reactions[a]



[a] The Fe^{III}–OOH species formed by the sequence **1** → **4** is converted into the oxo derivative **5**, which participates in hydroxylation and related reactions; however, with carbonyl substrate **4**, it is trapped to produce the adduct **6**, which undergoes an acyl-carbon fission. The wavy lines show the bonds broken during the latter reaction. In accordance with the accepted convention of inorganic chemistry while assigning the charge to iron in the structures above, it is assumed that the pair of electrons forming the coordination bond resides entirely with the ligand and is not shared with the metal.

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‡ In honor of his seventy-fifth birthday, this paper is dedicated to Professor Sir Derek Barton, the great guru of chemical sciences and the mentor of one of the authors (M.A.).

§ Deceased.

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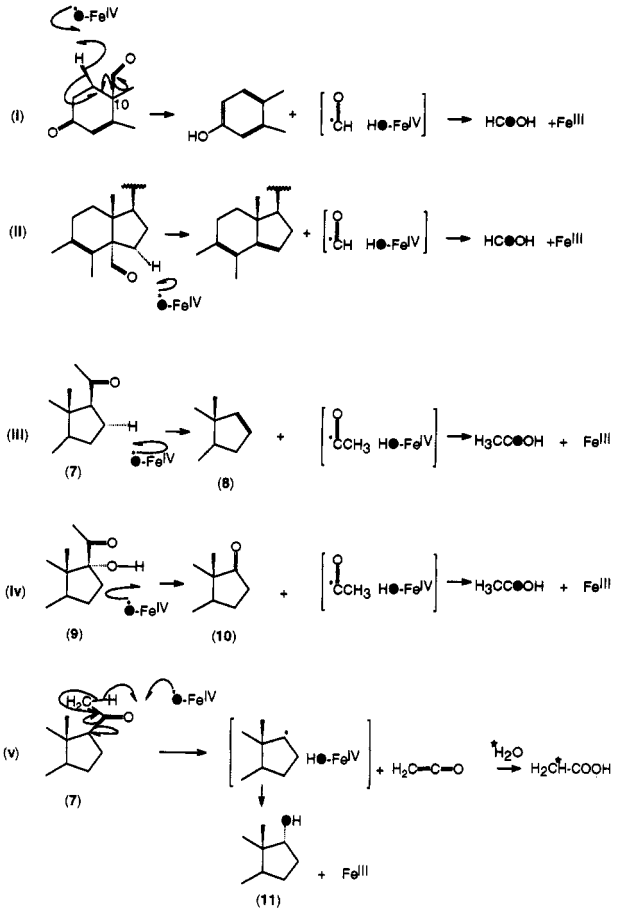
Groves, 1986; Akhtar & Wright, 1991). The intramolecular redox reaction **5a** → **5b** involved in the formation of the radical cation (**5b**) is presumably aided by the greater stability of Fe^{IV} over Fe^{V}. The $(\bullet+)Fe^{IV}{=}O$ species represented by one of its canonical forms, $Fe^{IV}{-}O^{\bullet}$ (**5c**), behaves like an alkoxy radical and participates in the hydroxylation reaction via a free radical mechanism, as shown by the formation of racemic (Groves *et al.*, 1978; Gelb *et al.*, 1982; White *et al.*, 1986) and rearranged products (Ortiz de Montellano & Stearns, 1987; Bowry *et al.*, 1989; Atkinson & Ingold, 1993).

Our past studies on certain steroid enzymes (aromatase, 14α-demethylase, and 17α-hydroxylase-17,20-lyase) have highlighted their multifunctional nature, suggesting that in these cases the same protein catalyst is involved in promoting reactions belonging to entirely different generic types (Akhtar *et al.*, 1977, 1978, 1982, 1993; Stevenson *et al.*, 1988; Wright & Akhtar, 1990; Cornia *et al.*, 1991; Miller *et al.*, 1991). These reactions are hydroxylation, oxidation of an alcohol into a carbonyl compound, and, most notably, an acyl-carbon cleavage reaction represented by the fragmentation path indicated in Scheme 1. We have suggested that these reactions are catalyzed at a single active site by two distinct iron–oxygen species: the oxo derivative (**5c**) being involved in hydroxylation and alcohol oxidation while an Fe^{III}–OOH species is involved in the acyl-carbon fission process (Akhtar *et al.*, 1982, 1993; Stevenson *et al.*, 1988; Akhtar & Wright, 1991; Corina *et al.*, 1991). Although the proposal has received impressive support initially from the groups of Robinson and Caspi (Cole & Robinson, 1988; Oh & Robinson, 1993; Ranjith *et al.*, 1993) and, more recently, from the findings of Coon, Vaz, and their colleagues (Roberts *et al.*, 1991; Vaz *et al.*, 1991), the alternative view that the acyl-carbon cleavage may also be promoted by the oxo derivative cannot be dismissed too lightly. As mentioned above, the oxo derivative is the key performer in the hydroxylation reaction and is also implicated in reactions catalyzed by catalase and a range of peroxidases. Why should not the same species be involved in the acyl-carbon cleavage process? Indeed, a mechanism using the oxo derivative has been considered for the C-10–C-19 bond cleavage in oestrogen biosynthesis (Beusen *et al.*, 1987; Korzekwa *et al.*, 1991). Let us consider the chemical consequences of involving the oxo derivative in a range of C–C bond cleavage reactions (Scheme 2). The first of these is catalyzed by aromatase, the second by 14α-demethylase, while the remaining three have been attributed to pig testes 17α-hydroxylase-17,20-lyase (P-$450_{17\alpha}$). Scheme 2 shows that, in four of the examples (panels i–iv), the mechanism involving the oxo derivative makes the same prediction as that using the iron peroxide, that is, the incorporation of an atom of oxygen from O_2 into the cleaved fragment. The formation of 17α-hydroxyandrogen (**11**, Scheme 2, panel v), however, poses an interesting problem. Since, as we shall see, in this case the C-16α, C-16β, and C-17α hydrogen atoms of the precursor are undisturbed in the overall conversion, if the oxo derivative were to engage in an initial hydrogen abstraction process, the only remaining position, that is C-21, must be involved. This feature is the main subject of this paper, in which we describe experiments providing information on the fate of C-16, C-17, and C-21 hydrogen atoms during the pregnene side-chain cleavage reactions. Furthermore, the incorporation of oxygen from O_2 into the side-chain, released as acetic acid, and isotopic analysis of steroid products are reported. The mechanistic implications of these findings are also discussed. A part of the work has been presented in a preliminary communication (Corina *et al.*, 1991).

Scheme 2: Theoretical Mechanisms for the Acyl-Carbon Fission Process Promoted by the Oxo Derivative[a]



[a] The reactions are catalyzed by the following enzymes: (i), 14α-demethylase; (ii), aromatase; (iii and iv), P-$450_{17\alpha}$; (v), probably P-$450_{17\alpha}$. ● is the oxygen derived from O_2.

EXPERIMENTAL SECTION

Chemicals were generally obtained from Aldrich Chemical Co. Ltd., Gillingham, Dorset, or Sigma Chemical Co., Poole, Dorset. 97% isotopically enriched $^{18}O_2$ admixed with argon to give a 1:2 (v/v) mixture was obtained from Isogas Ltd., Croydon, Surrey. $^{2}H_2$ having 99.5% enrichment of the isotope was obtained from Cambrian Gases, Croydon, Surrey. [7α-^{3}H]pregnenolone was obtained from New England Nuclear, and [17α,21-^{3}H]pregnenolone was prepared as described previously (Akhtar *et al.*, 1994).

Gas Chromatography–Mass Spectrometry (GC–MS). Isotopic distributions in the labelled substrates (**7a–f**) were measured by direct introduction probe MS analysis of the underivatized solid, since some exchange from labile positions was observed on GC. The chemical purities of all the steroidal substrates were determined by TLC and by GC–MS of the underivatized materials or their trimethylsilyl derivatives. All mass spectra were recorded in the electron impact positive-ion mode. The analysis of benzyl acetate (Figure 1), produced from enzymically liberated side chain as acetic acid, was performed as described elsewhere (Akhtar *et al.*, 1994). For the analysis of steroidal products from incubations, the material purified by TLC was taken to dryness and treated with a mixture of pyridine/bis(trimethylsilyl)trifluoroacetamide (1:1 v/v; 0.4 mL) at 50 °C for 15 min. An aliquot (1–2 μL) was analyzed under the following GC–MS conditions: column, 30 m × 0.25 mm i.d. capillary, phase 0.25 μ DB-5. The injector

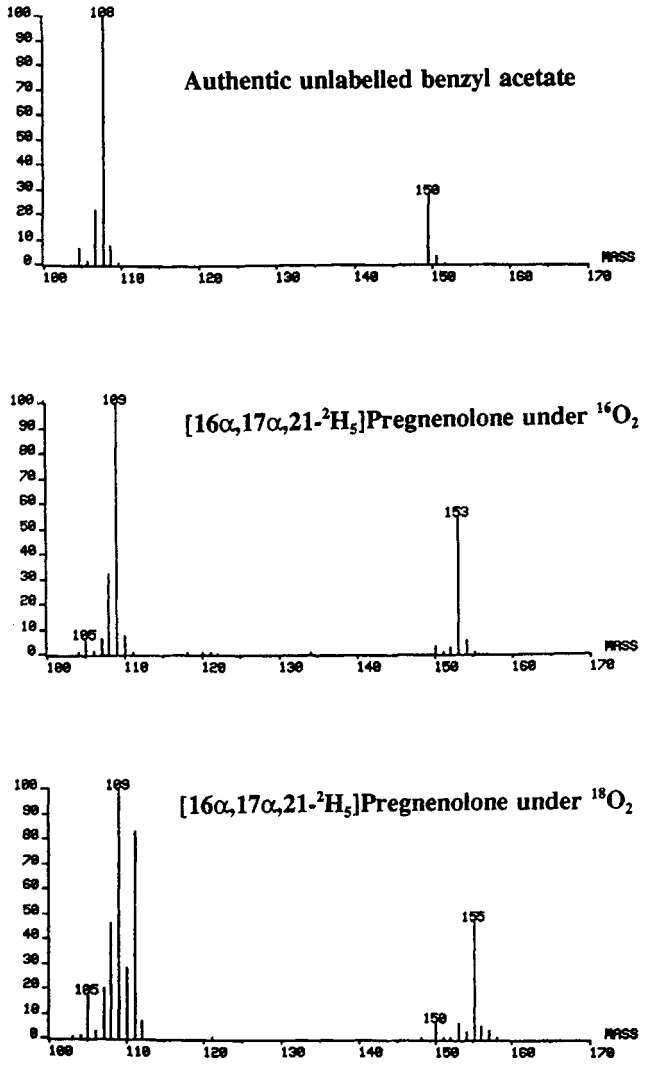


FIGURE 1: Mass spectra of authentic unlabeled benzyl acetate and of biosynthetic benzyl acetate obtained as indicated.

temperature was 250 °C, and the initial column temperature was maintained at 200 °C for 3 min and then programmed to increase 8 °C/min to 300 °C for 6 min, while the transferline temperature was 280 °C. The following retention times were recorded: androsta-5,16-dien-3β-ol trimethylsilyl ether, 7.5 min; androst-5-ene-3β-17α-diol bis(trimethylsilyl) ether, 9.5 min; and androst-5-ene-3β,17β-diol bis(trimethylsilyl) ether, 10.0 min. Single-ion recording (SIR) measurements on all three steroids were performed in the same chromatographic run by monitoring masses from 344.30 to 347.30 for androsta-5,16-dien-3β-ol trimethylsilyl ether (for the nonisotopic species, M^+, 344.30) and masses from 434.30 to 440.30 for androst-5-ene-3β,17α- or 17β-diol bis(trimethylsilyl) ether (for the nonisotopic species, M^+, 434.30). For unambiguous characterization, full spectra of GC peaks were also recorded from m/z 480 to 220 with a resolution of 1000 (Figure 2).

A mixture of known composition of the three steroids was also analyzed by SIR following the isotopically labeled determination to obtain semiquantitatively the ratios of the three key steroid products. For this purpose the intensities of the M^+ ions of the reference sample run were compared with the sum of the intensities of the M^+ ions of all the isotopomers of a given compound in the unknown mixture.

Preparation of Variously Deuteriated Isotopomers of Pregnenolone and Their Mass Spectral Data. [16α,17α-$^2H_2$]-Pregnenolone (**7d**) was prepared (Akhtar *et al.*, 1994) essentially by the method of Marker (Marker *et al.*, 1947) but using $^2H_2$ and had m/z (relative intensity) 316 (5), 317 (14), 318 (100), 319 (32), and 320 (8). [16α-^{2}H]Pregnenolone (**7a**) (Kohara & Shimizu, 1987) was obtained by the exchange of the 17α-deuterium from the preceding compound (**7d**) with 2.5% methanolic potassium hydroxide and had m/z (relative intensity) 316 (4), 317 (100), 318 (25), and 319 (5). [16β-^{2}H]Pregnenolone (**7b**) (Kohara & Shimizu, 1987) was prepared by converting [16α,16β-$^2H_2$]dehydroisoandrosterone into [16-^{2}H]3β-hydroxypregna-5,16-dien-20-one followed by reduction of the latter with H_2 and had m/z (relative intensity) 316 (7), 317 (100), and 318 (26). [16α,16β,17α-$^2H_3$]-Pregnenolone (**7e**) was prepared by the reduction of the preceding 5,16-diene with $^2H_2$ (Akhtar *et al.*, 1994) and had m/z (relative intensity) 318 (24), 319 (100), and 320 (37). Treatment of isotopomer **7e** with 2.5% methanolic potassium hydroxide gave [16α,16β-$^2H_2$]pregnenolone (**7c**), which had m/z (relative intensity) 317 (13), 318 (100), and 319 (37). [16α,17α,21-$^2H_5$]Pregnenolone (**7f**) was obtained from [16α,17α-$^2H_2$]pregnenolone (**7d**) by exchange with KO^{2}H/CH_3O^2H (2.5:100 w/v) and had m/z (relative intensity) 316 (3), 317 (0), 318 (4), 319 (11), 320 (23), and 321 (100). It is to be noted that the unlabeled **7** had m/z of 316.

Preparation of the Microsomal Fraction from Pig Testes, Incubation of Pregnenolone, and Isolation of Acetic Acid and the Steroidal Products. Fat-free testes (100–120 g) from 4–6 week old pigs were pooled and homogenized in 10 mM Tris-HCl buffer (195 mL, pH 7.4, containing 10 mM EDTA and 150 mM KCl) using a domestic blender. The homogenate was centrifuged at 10000*g* for 30 min. The supernatant was then spun at 105000*g* for 1 h, and following the resuspension of the resulting pellet in the above buffer (35 mL) by homogenization, the microsomal fraction was stored in 1.0-mL aliquots at −70 °C and contained 54 mg of protein/mL. The lyase activity of the enzyme was measured by the release of [^{3}H]H_2O plus [^{3}H]acetic acid from [17α,21-^{3}H]pregnenolone, and typically 1 mg of the microsomal protein catalyzed the cleavage of 0.5–0.8 mmol of pregnenolone/min.

In order to obtain sufficient material for a range of analyses, incubations were performed on a preparative scale as follows. A mixture of $NADP^+$ (5 mg), glucose 6-phosphate (10 mg), glucose 6-phosphate dehydrogenase (10 units) in 100 mM potassium phosphate buffer (6 mL) containing 1 mM EDTA, pH 7.25, and the microsomal fraction (4 mL containing 216 mg of protein) was rocked at 37 °C for 10–20 min. A solution of pregnenolone (1.0 mg), appropriately labeled with the heavy isotope, admixed with tracer amounts of [17α,21-^{3}H]pregnenolone (0.8 μCi) and [7α-^{3}H]pregnenolone (0.35 μCi) in dimethylformamide (50 μL), was then rapidly added to the incubation mixture, which was shaken at 37 °C for 75 min in air or under an atmosphere of $^{18}O_2$ (97% enrichment). Analysis of an aliquot showed that 30–45% of the original radioactivity was recovered in acetic acid plus water. For those experiments in which the analysis of the steroidal product as well as the side chain (released as acetic acid) was desired, the incubation mixture was divided into two halves. One half was processed to isolate acetic acid for mass spectrometric analysis (Akhtar *et al.*, 1994), and the second half of the incubation mixture (5 mL) was processed for the isolation of steroids as follows. After the addition of water (10 mL) the mixture was shaken with 10% methanol in ethyl acetate (3 × 15 mL). The combined organic extracts were washed with saturated brine (2 × 50 mL), dried with anhydrous sodium sulphate, and filtered. The filtrate was evaporated to dryness

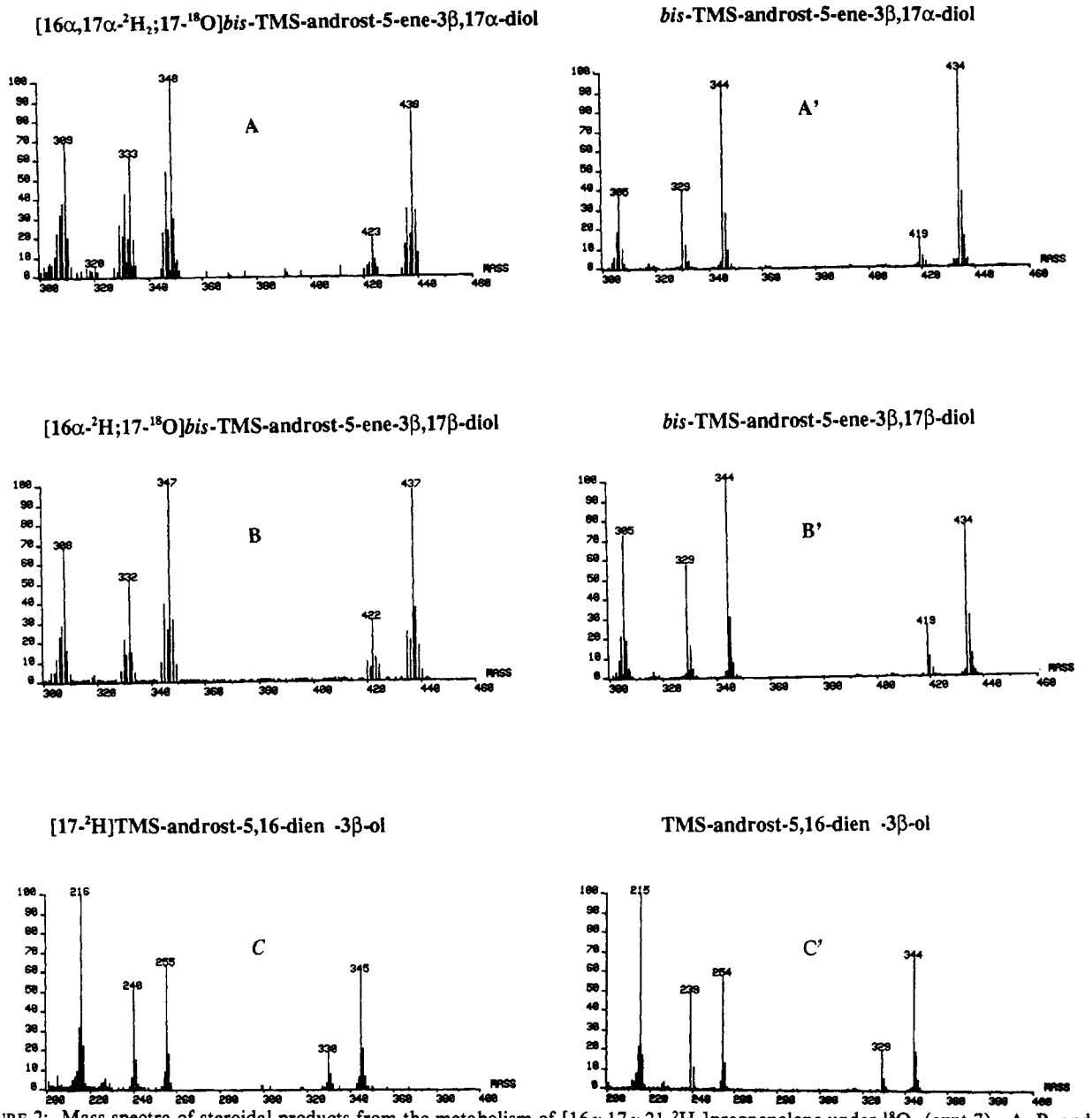


FIGURE 2: Mass spectra of steroidal products from the metabolism of [16α,17α,21-²H₅]pregnenolone under $^{18}O_2$ (expt 7). A, B, and C are the spectra of the TMS derivatives of biosynthetic compounds (**11**, **10**, and **8**). The compound **10** was analyzed following the reduction of the 17-ketone. A′, B′, and C′ are the corresponding spectra of authentic unlabeled compounds.

in vacuo. The residue was dissolved in methanol (5 mL) to which was added an excess of sodium borohydride. This mixture was allowed to stand at room temperature for approximately 1 h and then diluted with water (10 mL). The steroids were extracted in ethyl acetate (3 × 10 mL), and the organic layer washed with water (10 mL). Subsequent drying and evaporation, as before, gave a residue which was dissolved in dichloromethane and separated by preparative TLC (dichloromethane/acetone, 4:1 v/v). The steroids were located by scanning for radioactivity. Following extraction, the band containing a mixture of androsta-5,16-dien-3β-ol, androst-5-ene-3β,17β-diol, androst-5-ene-3β,17α-diol, and the unchanged precursor was analyzed by GC–MS as described above.

RESULTS

The overall strategy used in the present study may be considered in two parts. The first involved the delineation of the status of hydrogen atoms at the two nuclear positions, C-16 and C-17, during the conversion of pregnenolone into the three side-chain-cleaved steroids (**7** → **8**, **7** → **10**, and **7** → **11**, Scheme 3). In the second part, the study was extended to include the analysis of the released acetic acid.

Isotopic Analysis of Steroids Derived from Variously Deuteriated Samples of Pregnenolone. For the examination of the nuclear modifications, five samples of pregnenolone were prepared, and these contained deuterium atom(s) at the following positions: 16α (**7a**), 16β (**7b**), 16α, 16β (**7c**), 16α, 17α (**7d**), and 16α, 16β, 17α (**7e**). These samples were admixed with tracer amounts of pregnenolone labeled with ^3H in the side chain, so that the progress of the enzymic cleavage reaction may be conveniently monitored by measuring the ^3H radioactivity in the released acetic acid. Following a 75-min incubation, the steroidal fraction was extracted and treated with $NaBH_4$ to stabilize the exchangeable C-17 carbonyl oxygen of dehydroisoandrosterone, and following derivatization (with pyridine/bis(trimethylsilyl)trifluoroacetamide), the mixture was analyzed by GC–MS. The

Scheme 3: Status of Hydrogen and Oxygen Atoms in the Conversion of Pregnenolone into Various Steroids

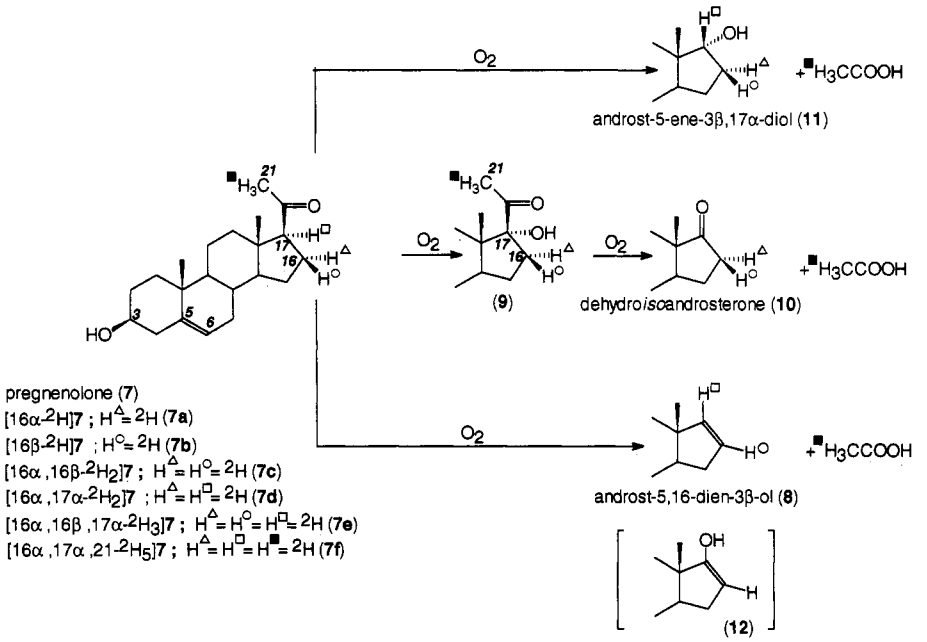


Table 1: Isotopic Composition of Steroids Obtained from the Incubation of Variously Deuteriated Pregnenolones under $^{18}O_2$ or $^{16}O_2$[a]

		% distribution of each isotopomer													
		adrosta-5,16-dien-3β-ol (**8**)			dehydroisoandrosterone (**10**)					androst-5-ene-3β,17α-diol (**11**)					
substrate and % isotopomer in the sample	gas phase	U	D_1	D_2	U	D_1	D_2 (or $^{18}O_1$)	[D_1; $^{18}O_1$]	[D_2; $^{18}O_1$]	U	D_1	D_2	[D_1; $^{18}O_1$]	[D_2; $^{18}O_1$]	[D_3; $^{18}O_1$]
1. [16α-²H]pregnenolone (**7a**) D_1 (96%); U (4%)	$^{18}O_2$	84	16	–	–	16	18	64		–	10	16	73		
2. [16β-²H]pregnenolone (**7b**) D_1 (93%); U (7%)	$^{18}O_2$	13	87		–	30	–	66		–	17	–	76		
3. [16α,16β²H₂]pregnenolone (**7c**) D_2 (86%); D_1 (10%); U (3%)	$^{18}O_2$	11	89		–	14	21[b]	8	53	–	–	11	9	79	
4. [16α,16β,17α-²H₃]pregnenolone (**7e**) D_3 (69%); D_2 (15%); D_1 (10%); U (6%)	$^{18}O_2$	–	15	81	–	16	34[b]	11	36	–	–	–	12	12	71
5. [16α,17α-²H₂]pregnenolone (**7d**) D_2 (84%); D_1 (12%); U (2%)	$^{18}O_2$	9	82	9	17	32	–	46		11	–	8	7	71	
6. [16α,17α,21-²H₅]pregnenolone (**7f**) D_5 (73%); D_4 (16%); D_3 (7%); U (2%)	$^{16}O_2$	5	94	–	17	83				–	–	100			
7. as in 6 above	$^{18}O_2$	10	87	–	5	23	12	60		–	–	10	–	78	

[a] Following the incubation, the mixture containing the steroidal products was treated with $NaBH_4$ and analyzed as the trimethylsilyl derivatives, as described in the Experimental Section. In calculating the percentage distribution, the contributions made by M + 1 and M + 2 ions have been taken into account and hence the values given are true percentages of the isotopomers. U denotes the unlabeled species whereas D_1, D_2, etc. are one-, two-, etc. deuterium-containing species, respectively. Isotopomers present in less than 5% of the total are denoted by a dash. The distribution of the three steroidal products in these experiments was as follows: **8** (51 ± 9%, n = 6), **10** (33 ± 8%, n = 6), and **11** (15 ± 4%, n = 6). [b] Whether these species contain two deuterium atoms or one atom of ^{18}O cannot be discerned from the mass spectral data.

cumulative data summarized in Table 1 confirm and extend several previous findings. The isotopic composition of dehydroisoandrosterone (**10**), analyzed as the bis-trimethylsilyl derivative of androst-5-ene-3β,17β-diol, showed the incorporation of oxygen from $^{18}O_2$ into the C-17 position (Table 1). The extent of labeling with ^{18}O varied between experiments, presumably due to variable exchange of the C-17 carbonyl oxygen of dehydroisoandrosterone with the oxygen of H_2O prior to the stabilization of the position with $NaBH_4$. The ^{18}O labeling of the 17-carbonyl group has been investigated previously, and the incorporation of 0.39 and 0.55 atom of ^{18}O was reported (Nakano *et al.*, 1968; Suhara *et al.*, 1984). In the present study, these values were improved and up to 0.66 atom of ^{18}O was found in **10** (Table 1, expts 1 and 2). A more significant finding with respect to the labeling profile of **10** is the demonstration that during its formation from [16α,16β-²H₂]pregnenolone (**7c**) both the C-16 hydrogen atoms of the precursor remain undisturbed (Table 1, expt 3; Scheme 3). The conservation of the two C-16 hydrogen atoms eliminates a mechanism for the side-chain cleavage in which dehydroisoandrosterone is formed by the rearrangement of an enolic intermediate of type **12**.

In the formation of the Δ^{16}-steroid **8**, the 17α-hydrogen of the precursor was retained but its 16α-hydrogen was removed (Table 1, expts 1, 2, and 4; Scheme 3), thus confirming the earlier observations (Shimizu & Nakada, 1976; Kohara & Shimizu, 1987; Corina *et al.*, 1991). We now consider the isotopic composition of 17α-hydroxyandrogen (**11**). When the latter compound was biosynthesized from [16α,17α-²H₂]-pregnenolone (**7d**), it was found to retain both the deuterium atoms of the precursor and incorporate 0.71 atom of ^{18}O (Table 1, expt 5). These results are in broad agreement with the previous findings (Shimizu, 1978, 1979). In the present study the use of pregnenolone labeled specifically with ²H at the C-16β position (Table 1, expt 2) or equally at both C-16 positions (α and β; Table 1, expt 3) gave the additional information that the 16β-hydrogen atom of the precursor also remained undisturbed, thus emphasizing that, during the

Table 2: Isotopic Composition of Benzyl Acetate Obtained from the Incubation of [16α,17α,21-$^2H_5$]Pregnenolone under $^{18}O_2$[a]

	relative intensities of the molecular ions due to the benzyl ester of					
expt	CH_3COOH (m/z 150)	CH_2DCOOH (m/z 151)	$CHD_2COOH + CH_3C^{18}OOH$ (m/z 152)	$CD_3COOH + CH_2DC^{18}OOH$ (m/z 153)	$CHD_2C^{18}OOH$ (m/z 154)	$CD_3C^{18}OOH$ (m/z 155)
1	36	0.8	1.2	12.5	5	100
2	101	1.2	1.4	4.7	14	100

[a] Several incubations were performed as described in the Experimental Section, and in each case half of the mixture was used for the isolation and derivatization of the side chain released as acetate. The results from two typical experiments are detailed. The other half was processed for the isolation of steroidal products, and their amounts were quantified by GC–MS. From four independent experiments, the distributions of the three steroids, androsta-5,16-dien-3β-ol (**8**), dehydroisoandrosterone (**10**), and androst-5-ene-3β,17α-diol (**11**) were found to be 61%, 22%, and 17%, respectively. D = ^2H.

formation of the 17α-hydroxyandrogen, none of the three neighboring hydrogen atoms at the 16α, 16β, or 17α positions are labilized. These findings highlight the fact that the 17α-hydroxyandrogen is not formed via the intermediacy of 17α-hydroxypregnenolone (**9**), since such a process will entail the loss of the 17α-deuterium; neither is it formed from the Δ^{16}-steroid **8**, which requires the loss of the 16α-deuterium nor by another unknown process in which the 16β-position might have been recruited. The salient features of our findings, showing the status of various hydrogen and oxygen atoms during these conversions, are shown in Scheme 3.

Isotopic Analysis of Acetate Released from CD_3-Labeled Pregnenolone Incubated under $^{18}O_2$. We next attended to the scrutiny of the hypothesis in which 17α-hydroxyandrogen (**11**) is biosynthesized involving the Fe^{IV}–O• species via the intermediacy of ketene, as shown in Scheme 2, panel v. For this purpose, pregnenolone labeled with deuterium in the 21-methyl group as well as in the two nuclear positions, 16α and 17α, was required. This (**7f**) was obtained by subjecting a sample of [16α,17α-$^2H_2$]pregnenolone (**7d**) to exchange with CH_3OD to incorporate three deuterium atoms at C-21. The mass spectral analysis of the resulting sample showed it to contain 70% of the desired D_5-isotopomer, in which C-21, C-16α, and C-17α were labeled with the isotopic hydrogen. The other predominant species constituting 15% and 7% of the mixture were the D_4- and D_3-isotopomers, respectively. From the mass spectral analysis, it was not possible to determine the precise distribution of the deuterium atoms in these isotopomers, but we shall see that the results obtained using the specimen are such that information on the positional location of the deuterium in the D_3- and D_4-species is not necessary.

The main focus of the study now was to determine the distribution of the various isotopomers of acetic acid formed when [16α,17α,21-$^2H_5$]pregnenolone (**7f**) was subjected to side-chain cleavage and in the same experiment to quantify the ratios of the three steroids produced. Over a 2-year period, more than a half a dozen independent incubations with different batches of pig testes microsomes and the D_5-pregnenolone, admixed with tracer amounts of [17α,21-^3H]-pregnenolone, were performed under $^{18}O_2$. From the radioactivity released in acetic acid plus water, it was estimated that in these experiments 30–40% of the precursor had undergone side-chain cleavage. The incubation mixtures were split into two: one was processed from the isolation of the steroid fraction while the other, following acidification, was freeze-dried and the distillate converted to benzyl acetate. The latter was analyzed by single-ion monitoring to quantify all the species of masses between m/z 150 ($CH_3COOBzl$) and 155 ($CD_3C^{18}OOBzl$) (Figure 1). The data from two typical analyses in Table 2 show that the isotopomers corresponding to $CHD_2COOBzl$ plus $CH_2DCO^{18}OBzl$ comprised only 1.2–1.4% of all the deuterium-containing species of acetic acid. The rationale for including only the deuteriated species in the calculation is that these species are the ones produced during the biosynthesis, while the nonisotopic benzyl acetate (m/z 150) arises from acetic acid ubiquitously present in reagents and enzyme preparations.

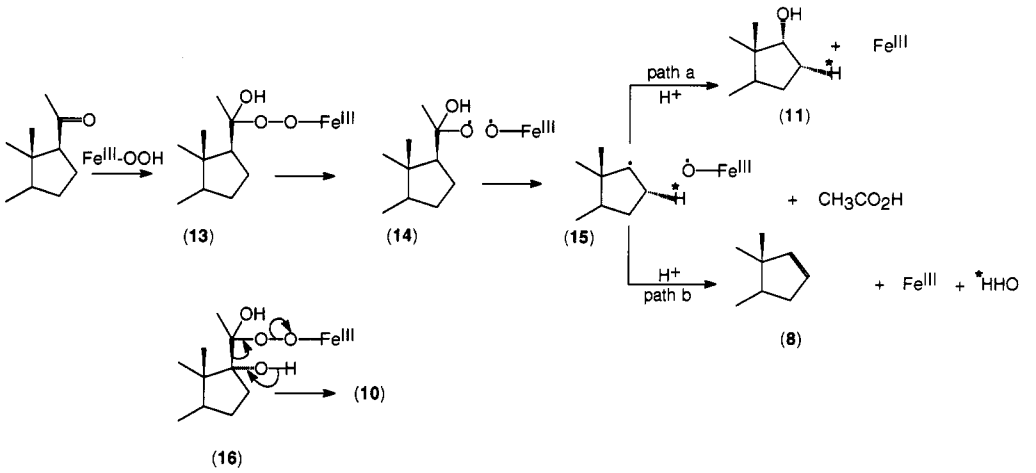
Evaluation of the Data in Terms of the Hypotheses Considered in Scheme 2, Panel v. The isotopic analysis of the steroid fractions showed that all three products had the expected labeling profile (Figure 2). Thus, the main isotopomer of dehydroisoandrosterone retained the C-16 deuterium of the precursor and had incorporated an atom of oxygen from $^{18}O_2$ (Table 1, expt 7), while 78% of 17α-hydroxyandrogen comprised the ^{18}O-containing isotopomer in which the two deuteriums located at C-16 and C-17 of the precursor were conserved. The labeling pattern of the Δ^{16}-steroid mirrored the results obtained in the first part of the study above as well as those reported by other workers (Shimizu, 1978, 1979).

The relative amounts of the three steroids (**8**, **10**, and **11**) produced in the enzymic incubation were determined from the single-ion scanning data of the relevant GC peak. The peak areas of all the molecular ions for a given compound were added and, following normalization, compared with each other. The analysis revealed that about 17% of the steroid mixture comprised 17α-hydroxyandrogen (**11**). If the side-chain cleavage reaction involved in the formation of compound **11** from [16α,17α,21-$^2H_5$]pregnenolone (**7f**) incubated under $^{18}O_2$ had occurred by the ketene path, Scheme 2, panel v, then an equivalent amount of the CHD_2COOH isotopomer would also have been present in the biosynthetic acetic acid. In the data of Table 2 we note that the molecular ion (m/z 152) due to the $CHD_2COOBzl$ species had the smallest intensity, which was less than 1.5% that of the most abundant ion (m/z 155) of $CD_3CO^{18}OBzl$. It should also be noted that, among the D_2-isotopomers, the one containing ^{18}O (m/z 154) was the significant species. These results thus highlight that in stoichiometric terms, $CHD_2COOBzl$ was present in less than 1/10th the amount of 17α-hydroxyandrogen. We thus conclude that the latter compound (**11**) could not have arisen from the ketene path and that its genesis involves a mechanism in which all three C-21 deuterium atoms of the precursor are retained in acetic acid and one of its oxygen atoms is derived from $^{18}O_2$, precisely the same isotopic pattern as was deduced for acetic acid released during the formation of the two other companion steroids **8** and **10** (Akhtar *et al.*, 1994).

DISCUSSION AND CONCLUSIONS

Cloning experiments (Zuber *et al.*, 1986; Namiki *et al.*, 1988) and enzyme purification studies (Nakajin *et al.*, 1981; Suhara *et al.*, 1984; Perrin *et al.*, 1991) have established that P-450$_{17\alpha}$ from several mammalian sources are monomeric species and catalyze the sequential conversion of progestrogens

Scheme 4: Mechanism for the Cleavage of the Pregnene Side Chain via a Free Radical (Upper Sequence) or an Ionic Mechanism (**16** → **10**)



into androgens by a two-step process, **7** → **9** → **10**, involving hydroxylation and C–C bond cleavage reactions. In addition, P-450$_{17\alpha}$s from pig and human testes produce the 5,16-diene **8** from pregnenolone (**7**) and are also suggested to catalyze the unusual conversion of the latter (**7**) into 17α-hydroxyandrogen (**11**) (Shimizu, 1978; Nakajin *et al.*, 1985; Weusten *et al.*, 1989).

The main thrust of the present study was to critically examine the salient mechanistic features of Scheme 1. The proposal therein restates the currently accepted view that in the conventional P-450 enzymes the Fe^{III}–OOH intermediate formed by the sequence **1** → **4**, Scheme 1, is converted into the oxo derivative **5**, which then participates in hydroxylation, epoxidation, and related reactions. The novel feature of our proposal, however, is that, in the case of multifunctional P-450s (aromatase, 14α-demethylase, and the pregnene side-chain cleavage system) which also catalyze an acyl-carbon bond cleavage, the Fe^{III}–OOH intermediate is trapped by the electrophilic property of the carbonyl group to produce a peroxy adduct (**6**) that decomposes to give the products according to the fragmentation path in Scheme 1. Notwithstanding this, a strong contestant to the mechanism is an alternative in which the acyl-carbon bond cleavage is also promoted by the oxo derivatives **5**. We have examined the consequences of using the Fe^{IV}–O$^\bullet$ species in five C–C bond cleavage reactions and have noted that, in four of the cases (Scheme 2), the two mechanisms (using either Fe^{III}–OOH or Fe^{IV}–O$^\bullet$) predict the same outcome; that is, the loss of the hydrogen atom from the α-position with respect to the scissile bond and the incorporation of an atom of oxygen from O_2 into one of the cleaved fragments. The fifth example, involving the formation of the 17α-hydroxyandrogen (**11**), poses an interesting problem. The cumulative information provided by the present work (Scheme 3), and reported elsewhere, highlights that in this case the three hydrogen atoms resident at positions 16α, 16β, and 17α of the precursor are retained in the product **11**. Therefore, for the Fe^{IV}–O$^\bullet$ intermediate to promote a fragmentation process, the initial reaction must involve the abstraction of one of the C-21 hydrogen atoms, generating the C-21 carbon radical, which may fragment to the C-17 radical. The latter ultimately produces the 17α-hydroxyandrogen by an oxygen-rebound process (Scheme 2, panel v). This scenario requires that the side chain is initially expelled as ketene and then converted to acetate by hydration. The mechanism therefore predicts that when pregnenolone containing CD_3 at position 21 is subjected to side-chain cleavage in an atmosphere of $^{18}O_2$, the CHD_2COOH isotopomer is produced in an amount stoichiometric with that of 17α-hydroxyandrogen (**11**). A detailed study reported here now shows that this is not the case and the CHD_2COOH isotopomer, if formed during the side-chain cleavage process, is present in less than 1/10th the amount of 17α-hydroxyandrogen. The status of the various hydrogen and oxygen atoms defined in this paper therefore eliminates the mechanism for the formation of the 17α-hydroxyandrogen that may operate via a Fe^{IV}–O$^\bullet$ intermediate.

The most important conclusions to be drawn from these results are that the introduction of the 17α-hydroxyl group in **11** occurs solely at the expense of the cleavage of the C-17–C-20 bond and that the overall process corresponds to an inversion of configuration. *The results forcefully highlight that the conversion must occur by a stepwise process.* Although the nature of the intermediates which may participate in the conversion is not directly revealed by our experiments, chemical considerations dictate that these should be radical species. These features are most readily accommodated by extending the mechanism considered previously for aromatase (see citations in the introduction) to the pregnene side-chain cleavage reaction, producing 17α-hydroxyandrogen (**11**) as follows. The initial event in the process is the formation of adduct **13** (Scheme 4), which decomposes by a radical mechanism to give acetate and the C-17 radical species **15**. The formation of 17α-hydroxyandrogen from the carbon radical **15** then occurs by an oxygen-rebound process (Scheme 4, path a). The merit of this proposal is that it can be extended to the genesis of the Δ^{16}-steroid, which may be produced from the same carbon radical by the loss of the 16α-hydrogen atom (path b). The two modes for the neutralization of the carbon radical are in keeping with the expected chemical behavior of such a species in nonenzymic systems. Another point to note is that in both cases the bonding events occur from the α-face of the steroid skeleton, which indicates, but does not prove, that the two products may be produced at the same active site through the loss of stringent control by the enzyme.

In our view, the formation of 17α-hydroxyandrogen, as shown in Scheme 4, is at present the strongest evidence available for the participation of a peroxy adduct, i.e. **13**, in a C–C bond cleavage process. This conclusion is valid whether the formation of 17α-hydroxyandrogen is catalyzed by 17α-hydroxylase-17,20-lyase or another enzyme. Should the three cleavage reactions (Scheme 3) turn out to be the property of the same enzyme, then the justification for invoking the participation of a peroxy adduct in the other two transformations **9** → **10** and **7** → **8** will be very strong indeed. When the mechanistic principle is applied to the conversion of 17α-

hydroxypregnenolone (**9**) into the 17-ketosteroid **10**, then a point to note is that the equivalent peroxy adduct **16** (Scheme 4) could decompose by an ionic mechanism because the system now contains a readily ionizable O–H bond. *In this case the necessity of invoking a radical process is not as paramount as in those fragmentations where the C–H bond to be broken has a high pK value (>40)*, as in the case of the reactions **7** → **11** and **7** → **8**.

Further support for our original proposal that a peroxide mechanism may promote an acyl-carbon cleavage is provided by several other findings. First, Caspi and co-workers during their studies on aromatase have isolated minor products whose formation are readily explained by rearrangements of the C-10 radical (Ranjith *et al.*, 1993), which in turn is most conveniently produced from the decomposition of a C-19 peroxy species. Secondly, in model studies, Robinson's group has shown that the decomposition of a synthetic 19-hydroxy 19-hydroperoxide leads to formation of an aromatic compound (Cole & Robinson, 1988). Thirdly, the recent work of Vaz and Coon examined the metabolism of a large number of aliphatic aldehydes with eight different drug metabolizing P-450 enzymes (Roberts *et al.*, 1991; Vaz *et al.*, 1991). It was found that four of these were capable of catalyzing the acyl-carbon fission of Scheme 1, as exemplified by the conversion of cyclohexylaldehyde into cyclohexene. With these enzymes the oxo derivative **5** generated artificially was ineffective in the cleavage reaction but the P-450s in the presence of H_2O_2 gave cyclohexene and formate. The latter reaction may be rationalized by invoking the formation of an Fe^{III}–OOH intermediate, from the Fe^{III} form of the enzyme and H_2O_2, which then participates in the fragmentation process. Finally, during the reactions catalyzed by 14α-demethylase and P-450$_{17\alpha}$, O-acyl derivatives are produced: genesis of these compounds is best rationalized by invoking the Baeyer–Villiger rearrangements of corresponding peroxides (Fischer *et al.*, 1991; Mak & Swinney, 1992). In our original mechanistic analysis the possibility that peroxides of type **6** may undergo a 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). Irrespective of whether the rearranged compounds cited above arise from side reactions or are the main products, their isolation is tell-tale evidence that peroxides may be involved during the catalytic cycles of these P-450s.

In conclusion, we have scrutinized the proposal originally made in connection with aromatase (P-450$_{arom}$) that in the case of multifunctional steroidal P-450s the electronic structure of the sensitive C-atom in the substrate determines the course of the overall reaction by selecting one of the compatible oxygen species, **4** or **5**. The results now obtained on the stereochemistry as well as the status of various oxygen and hydrogen atoms in the formation of 17α-hydroxyandrogen (**11**) from pregnenolone (**7**) have strengthened this possibility, showing that the acyl-carbon cleavage process involved in the conversion may occur using the Fe^{III}–OOH species. That the catalytic duality shown in Scheme 1 is not confined to the steroidal enzymes but may be a phenomenon of wider occurrence is indicated by the reaction catalyzed by nitric oxide synthase (Moncada *et al.*, 1991; Kwon *et al.*, 1990; Leone *et al.*, 1991). In this case also, the enzyme carries out multicatalysis in which the first step is a hydroxylation reaction followed by a fragmentation process which may be regarded as the cleavage of an azaacyl–nitrogen bond. The azaacyl–nitrogen (C(=NH)N) group is isoelectronic with the acyl-carbon functionality of Scheme 1 and could trap the Fe^{III}–OOH species, producing an adduct whose mode of fragmentation may be modeled (White & Marletta, 1992) on the one we had originally proposed for aromatase and related enzymes (Akhtar *et al.*, 1977, 1978, 1982, 1993; Stevenson *et al.*, 1988; Wright & Akhtar, 1990; Corina *et al.*, 1991; Miller *et al.*, 1981).

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