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Metabolism of 19-Methyl-Substituted Steroids by Human Placental Aromatase†

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ABSTRACT: The 19-methyl analogues of androstenedione and its aromatization intermediates (19-hydroxyandrostenedione and 19-oxoandrostenedione) were evaluated as substrates of microsomal aromatase in order to determine the effect of a 19-alkyl substituent on the enzyme's regiospecificity. Neither the androstenedione analogue [10-ethylestr-4-ene-3,17-dione (**1c**)] nor the 19-oxoandrostenedione analogue [10-acetylestr-4-ene-3,17-dione (**3c**)] was converted to estrogens or oxygenated metabolites by placental microsomes. In contrast, both analogues of 19-hydroxyandrostenedione [10-[(1*S*)-1-hydroxyethyl]estr-4-ene-3,17-dione (**2c**) and 10-[(1*R*)-1-hydroxyethyl]estr-4-ene-3,17-dione (**2e**)] were converted to the intermediate analogue **3c** in a process requiring O_2 and either NADH or NADPH. No change in enzyme regiospecificity was detected. The absolute configuration of **2e** was determined by X-ray crystallography. Experiments with $^{18}O_2$ established that **3c** generated from **2c** retained little ^{18}O (<3%), while **3c** arising from **2e** retained a significant amount of ^{18}O (≈70%). All four 19-methyl steroids elicited type I difference spectra from placental microsomes in addition to acting as competitive inhibitors of aromatase (K_I = 81 nM, 11 μM, 9.9 μM, and 150 nM for **1c**, **2c**, **2e**, and **3c**, respectively). Pretreatment of microsomes with 4-hydroxyandrostenedione (a suicide inactivator of aromatase) abolished the metabolism of **2c** and **2e** to **3c**, as well as the type I difference spectrum elicited by **2c** and **2e**. The failure of **2c**, **2e**, and **3c** to undergo aromatization was rationalized in the context of a mechanistic proposal for the third oxygenation of aromatase requiring hydrogen abstraction at C_1 of 19,19-dihydroxyandrostenedione, homolytic cleavage of the C_{10}–C_{19} bond, and oxygen rebound at C_{19}.

Aromatase is a microsomal P-450 that executes three successive monooxygenations on androstenedione (**1a**) to generate in order 19-hydroxyandrostenedione (**2a**), 19-oxoandrostenedione (**3a**), and the products estrone (**4a**) and formic acid (Meyer, 1955; Ryan, 1959; Longchampt et al., 1960; Morato et al., 1961; Wilcox & Engel, 1965; Akhtar & Skinner, 1968; Axelrod et al., 1965; Starka & Breuer, 1970; Thompson & Siiteri, 1974a,b). An analogue of androstenedione in which a C_{19} hydrogen has been replaced by an acetylene (**1b**) acts as a suicide inactivator of aromatase (Covey et al., 1981; Metcalf et al., 1981; Marcotte & Robinson, 1982), although its mechanism in doing so is unknown. By executing its normal catalytic function, aromatase could generate an acetylenic ketone via the sequence **1b** → **2b** → **3b**. Michael addition of an enzyme nucleophile to **3b** would result in the formation of

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adduct **5** with subsequent loss of enzymatic activity. Alternatively, aromatase could insert oxygen directly into the acetylene of **1b** with eventual formation of the heme adduct **6** and inactivation of aromatase in a process similar to that documented for other P-450 enzymes (Ortiz de Montellano & Correia, 1983).

A significant difference between these two mechanisms is in the regiochemistry of aromatase oxygenation, with the first requiring oxygenation at C_{19} and the second requiring oxygenation of the acetylene adjacent to C_{19}.[1] The implication in this second mechanism that a 19-acetylene can change the regiospecificity of aromatase led to the question of what effect a 19-alkyl substituent might have on the enzyme's regiospecificity. The simplest such analogue of androstenedione, 10-ethylestr-4-ene-3,17-dione (**1c**), has one of the C_{19} hydrogens replaced by a methyl group. Oxygenation of **1c** by aromatase with the normal regiospecificity could give rise initially to **2c** or **2e** and subsequently to **3c**, each of which is analogous to normal aromatization intermediates. Alternatively, aromatase oxygenation of **1c** could result in compound **7** if the C_{19} substituent were to alter the regiospecificity of aromatase. To probe this possibility, compounds **1c**, **2c**, **2e**, and **3c** were synthesized, and their metabolism by microsomal aromatase was determined.

Experimental Procedures

Materials. The 4-[1,2-^{3}H(N)]androstene-3,17-dione (56.0 Ci/mmol) was purchased from New England Nuclear. The NAD, NADH, NADP, NADPH, and miconazole were purchased from Sigma. Human placental microsomes were prepared and assayed as described previously (Covey et al., 1981) and had specific activities ranging from 210 to 501 pmol of estrogen min^{-1} (mg of protein)$^{-1}$. Protein determinations were done with a Bio-Rad Protein Assay Kit I obtained from Bio-Rad Laboratories. Gas chromatographic supplies were obtained from Supelco, Inc. The 19-hydroxyandrostenedione was purchased from G. D. Searle, Inc. The Woelm silica gel dry column grade was purchased from Universal Scientific, Inc. Silica gel GF precoated glass thin-layer chromatography plates were from Analtech, Inc. HPLC solvents were obtained from Mallinckrodt, Inc., and methylmagnesium bromide was purchased from the Alpha division of Ventron Corp. The 4-hydroxyandrostenedione was prepared as described earlier (Brodie et al., 1981). $H_2{}^{18}O$ (98% ^{18}O) and $^{18}O_2$ (98% ^{18}O) were obtained from Cambridge Isotope Laboratories. Elemental analyses were performed by Galbraith Laboratories, Inc. Melting points were determined on a Kofler micro hot stage and are uncorrected. Proton magnetic resonance spectra were recorded in [^{2}H]chloroform on a Varian XL-300 spectrometer. Infrared spectra were recorded in KBr on a Perkin-Elmer 710B spectrometer or as a film on NaCl plates on a Perkin-Elmer 1710 Fourier transform spectrometer. HPLC was performed using a Waters Associates chromatograph and refractive index detector. Incubation buffer consisted of 10 mM potassium phosphate, pH 7.5, 100 mM KCl, and 1 mM EDTA.[2]

10-Ethylestr-4-ene-3,17-dione (**1c**). This compound was prepared by the method of Halpern et al. (1966) and recrystallized from acetone/hexane: mp 101–105.5 °C; IR (KBr) 1740 (C=O), 1675 (C=O), and 1620 cm^{-1} (C=C); NMR ($CDCl_3$) δ 0.88–0.93 (t overlapping s, 6, 10-CH_3CH$_2$– and CH_3, C-18), 5.92 (s, 1, H—C=C); λ_{max} (EtOH) 241 nm, ϵ = 17 000 M^{-1} cm^{-1}; mass spectrum, *m/e* 300 (M^+).

10-[(1S)-1-Hydroxyethyl]estr-4-ene-3,17-dione and 10-[(1R)-1-Hydroxyethyl]estr-4-ene-3,17-dione (**2c** *and* **2e**). Using the method of Iriarte et al. (1963), 3,17-bis(ethylenedioxy)androst-5-en-19-al was prepared from 19-hydroxyandrostenedione. The 3,17-bis(ethylenedioxy)androst-5-en-19-al (920 mg, 2.4 mmol) in dry tetrahydrofuran (25 mL) was added dropwise to a stirred solution of methylmagnesium bromide (10 mL of a 2.9 M solution in Et_2O) in dry tetrahydrofuran (50 mL). Once the addition was complete, the reaction was refluxed for 30 min. After cooling, several drops of 5% NH_4Cl and 100 mL of ethyl acetate were added, and the reaction was filtered through a medium sintered glass funnel. The filtrate was washed 3 times with water (30 mL). The organic layer was dried over Na_2SO_4 and evaporated to an oil, which crystallized on standing. The two major products were purified by HPLC on an Altex ultrasphere-Si column (5 μm, 10 mm × 25 cm, 10% acetone in hexane, 4.5 mL/min). Retention times and yields of the two products were as follows: 10-[(1*S*)-1-hydroxyethyl]-3,17-bis(ethylenedioxy)estr-5-ene, 13.1 min (471 mg, 49.2%); 10-[(1*R*)-1-hydroxyethyl]-3,17-bis(ethylenedioxy)estr-5-ene, 18.0 min (158 mg, 16.5%).

The *S* isomer was stirred at room temperature for 6 h in acetone (50 mL) and 10% H_2SO_4 in water (5 mL). The reaction was neutralized to pH 7 with 5% $NaHCO_3$ and extracted 3 times with methylene chloride. The organic layer was dried over Na_2SO_4 and evaporated to an oil. The desired product, **2c**, was isolated by chromatography on silica gel (eluant 1:1 benzene/ethyl acetate) and recrystallized twice from acetone/hexane: yield 206 mg; mp 197.0–201.0 °C; IR (KBr) 3475 (OH), 1740 (C=O), 1655 (C=O), and 1620 cm^{-1} (C=C); NMR ($CDCl_3$) δ 0.95 (s, 3, CH_3, C-18), 1.21 (d, 3, 10-CH_3CHOH–), 4.48 (m, 1, C*H*OH), 5.87 (d, 1, H—C=C); λ_{max} (EtOH) 245 nm, ϵ = 15 000 M^{-1} cm^{-1}; mass spectrum, *m/e* 358 (M^+, acetylated). Anal. Calcd for $C_{20}H_{28}O_3$: C, 75.91; H, 8.92. Found: C, 76.26; H, 8.64.

The *R* isomer was treated in a similar fashion to yield **2e**: yield 32 mg; mp 150.5–154.5 °C; IR (KBr) 3470 (OH), 1740 (C=O), 1655 (C=O), and 1615 cm^{-1} (C=C); NMR ($CDCl_3$) δ 0.94 (s, 3, CH_3, C-18), 1.39 (d, 3, 10-CH_3CHOH), 4.27 (m, 1, C*H*OH), 5.97 (d, 1, H—C=C); λ_{max} (EtOH) 244 nm, ϵ = 14 900 M^{-1} cm^{-1}; mass spectrum, *m/e* 358 (M^+, acetylated). Anal. Calcd for $C_{20}H_{28}O_3$: C, 75.91; H, 8.92. Found: C, 76.07; H, 8.93.

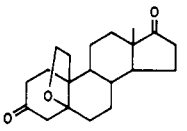
10-Acetylestr-4-ene-3,17-dione (**3c**). A mixture of **2c** and **2e** was oxidized with Jones reagent to yield the desired product, which was recrystallized from ethyl acetate/hexane: mp 114.5–115.0 °C; IR (KBr) 1745 (C=O), 1700 (sh, C=O), 1670 (C=O), and 1620 cm^{-1} (C=C); NMR ($CDCl_3$) δ 0.96 (s, 3, CH_3, C-18), 2.26 (s, 3, acetate CH_3), 5.96 (s, 1, H—C=C); λ_{max} (EtOH) 247 nm, ϵ = 11 900 M^{-1} cm^{-1}; mass spectrum, *m/e* 314 (M^+). Anal. Calcd for $C_{20}H_{26}O_3$: C, 76.40; H, 8.34. Found: C, 76.36; H, 8.26.

Attempted Preparation of 10-(2-Hydroxyethyl)estr-4-ene-3,17-dione (**7**). 5α-Bromo-6β,19-epoxyandrostane-3β,17β-diol diacetate was cleaved with zinc and oxidized with pyridinium chlorochromate to yield 19-oxoandrost-5-ene-3β,17β-diol diacetate. From this product, 19-formyl-5-androstene-3β,17β-diol diacetate was prepared by the method of Marcotte and

[1] IUPAC rules indicate that only a methyl group attached to C_{10} is correctly numbered as C_{19}. Larger alkyl groups attached to C_{10} are considered as C_{10} side chains and numbered accordingly. For purposes of clarity, however, the carbon atom attached to C_{10} is referred to as C_{19} in the text even when it is part of a larger alkyl group.

[2] Abbreviations: 4-OHA, 4-hydroxyandrost-4-ene-3,17-dione; 17β-HSD, 17β-hydroxysteroid dehydrogenase; GC–MS, gas chromatography–mass spectrometry; SIM, selected ion monitoring; EDTA, ethylenediaminetetraacetic acid.

Robinson (1982). Conversion of this steroid to 10-[2-[(*tert*-butyldimethylsilyl)oxy]ethyl]estr-4-ene-3,17-dione was effected by (1) $NaBH_4$ reduction of the 19-formyl group; (2) reaction with *tert*-butyldimethylsilyl chloride and imidazole in dimethylformamide, (3) cleavage of the 3β- and 17β-acetates with KOH–methanol, and (4) Oppenauer oxidation. Removal of the silyl protecting group was attempted with $N(Bu)_4^+F^-$ in tetrahydrofuran, HF in CH_3CN, Amberlite IR-120-P in methanol, and $BF_3{\cdot}Et_2O$ in $CHCl_3$, but the desired product was not observed. Instead, the cyclic ether resulting from intramolecular addition of the deprotected 10-(2-hydroxyethyl) group to the Δ^4-3-one system was isolated, 5,10-(oxyethylene)-5β-estrane-3,17-dione (**8**): mp 172.5–176.5 °C; IR 1734 (C=O) and 1716 cm^{-1} (C=O); NMR ($CDCl_3$) δ 0.92 (s, 3, CH_3), 3.90–3.99 (m, 2, $-CH_2-O$); UV none; mass spectrum, *m/e* 316 (M^+). Anal. Calcd for $C_{20}H_{28}O_3$: C, 75.91; H, 8.92. Found: C, 76.02; H, 8.84.



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Deprotection of the silyl steroid in 3:1:1 acetic acid/water/tetrahydrofuran led to initial formation of the desired product, which with time went on to form the cyclic ether. The 10-(2-hydroxyethyl)estr-4-ene-3,17-dione could be trapped as its acetate by addition of an excess of 1:1 Ac_2O/pyridine at ≈1 h of reaction and purification by HPLC (10-μm silica, 20% acetone in hexane, 2.8 mL/min, retention time 14.5 min, using a Waters Associates radial compression module): IR 1737 (C=O), 1671 (C=O), and 1615 cm^{-1} (C=C); NMR ($CDCl_3$) δ 0.93 (s, 3, CH_3, C-18), 2.04 (s, 3, acetate CH_3), 4.0–4.2 (m, 2, $-CH_2-O$), 5.91 (s, 1, H—C=C); λ_{max} (EtOH) 240 nm, ϵ = 12 900 M^{-1} cm^{-1}; mass spectrum, *m/e* 358 (M^+, acetylated).

Crystallographic Studies. Crystals of 10-[(1*R*)-1-hydroxyethyl]estr-4-ene-3,17-dione ($C_{20}H_{28}O_3$, **2e**) are monoclinic with a = 14.735 (3) Å, b = 6.455 (2) Å, c = 9.266 (2) Å, and β = 101.14 (2)°. The space group is $P2_1$ with Z = 2 molecules of FW 316.44, and the calculated density is 1.216 cm^{-3}. Three-dimensional X-ray diffraction data were collected on a Syntex $P2_1$ four-circle diffractometer with Cu Kα radiation (λ = 1.5418 Å) utilizing a graphite monochromator. The data were measured with the $\theta - 2\theta$ technique to a maximum 2θ = 138° ($\sin\theta/\lambda_{max}$ = 0.602 $Å^{-1}$). A total of 1776 independent reflections were measured of which 1564 had $I > 1.5\sigma$ and were considered as observed. The data were corrected for Lorentz and polarization effects; however, no correction for absorption was done.

The structure was determined by direct methods with MULTAN80 (Main et al., 1980) and was refined by crystallographic least squares with the program ICRFMLS (Carrell, 1969). For the final refinement, all non-hydrogen atoms were refined with anisotropic temperature factors while the hydrogen atoms were refined with isotropic temperature factors. The final agreement indices were R = 0.037 and R_w = 0.042, where $R = \sum||F_o| - |F_c||/\sum|F_{ob}|$ and $R_w = [\sum_w(|F_o| - |F_c|)^2/\sum w|F_o|^2]^{1/2}$. The observed structure amplitude is represented by F_o while the calculated value is F_c and w is the least-squares weight defined by the relation $w = 1/\sigma^2(F_o)$ for data with $I > 1.5\sigma(I)$ and $w = 0$ for data with $I < 1.5\sigma(I)$. The results of the structure determination are illustrated in Figure 1, along with some pertinent bond lengths. The identity of the hydroxyl group has been unambiguously established in this study by molecular geometry and by crystal packing,

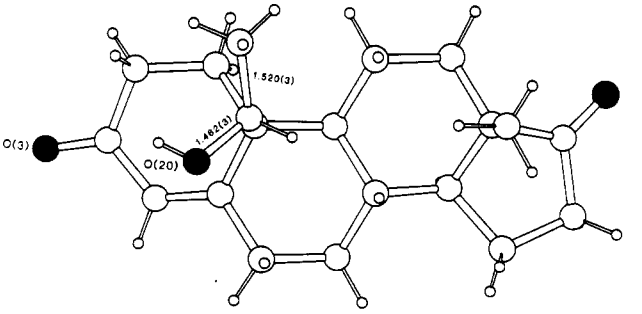


FIGURE 1: β-Face view of the crystal structure of 10-[(1*R*)-1-hydroxyethyl]estr-4-ene-3,17-dione (**2e**). This diagram was produced by the program VIEW (Carrell, 1976).

which clearly indicates hydrogen bonding between the hydroxyl group O(20)–H(20) on one molecule and O(3) on a second molecule forming an infinite chain of molecules linked in this manner. The molecule clearly is established as the *R* isomer **2e**.

Competitive Inhibition Studies. Competition experiments were carried out as described previously (Covey et al., 1981). Concentrations of inhibitors evaluated were 50 μM and 100 μM for **2c** and **2e** and 0.5 μM and 1.0 μM for **1c** and **3c**. Kinetics results were analyzed by Lineweaver–Burk plots (1934).

Incubation of Steroids with Placental Microsomes and Isolation of Products. Incubations (9.0 mL) in 50-mL Erlenmeyer flasks contained 27 mg of microsomal protein, steroid (1.8 μmol in 0.18 mL of ethanol), and incubation buffer. Each incubation contained either NAD, NADH, NADP, or NADPH (10 mM) and was shaken at 37 °C for 1 h. Products were isolated as described previously (Beusen et al., 1986). The steroidal products were taken up in 200 μL of CH_2Cl_2, and 2 μL was spotted on a silica gel GF TLC plate (100 × 25 mm, 250 μm thickness). The plate was run in 1:1 benzene/ethyl acetate and visualized by UV and by charring after spraying with 10% H_2SO_4 in methanol. The remaining steroids in CH_2Cl_2 were applied to a small silica gel column (55 mm × 5 mm) and eluted in 1-mL fractions with 40% ethyl acetate in hexane.

Preparation of Aromatase-Inactivated Microsomes. Microsomal incubations with 4-OHA (180 nmol in 180 μL of ethanol) and NADPH (10 mM) were set up as described above. Controls, run in parallel, contained ethanol without 4-OHA. Aromatase activity was determined prior to addition of 4-OHA (or ethanol) and following 35 min of incubation according to the method of Covey and Hood (1982). Microsomes were pelleted (100000*g*, 60 min, 4 °C) after 35 min of incubation, and supernatants were discarded. For metabolism studies, pellets were resuspended in incubation buffer to a protein concentration of ≈3 mg/mL. For difference spectroscopy, pellets were resuspended in incubation buffer to a protein concentration of ≈1.2 mg/mL. Following resuspension, aliquots were removed for protein and activity assays.

Incubation of **2c** *and* **2e** *with Placental Microsomes in the Absence of Oxygen.* Microsomes (18 mg of protein), NADPH (10 mM), and buffer were placed in 25-mL Erlenmeyer flasks (total volume 5.82 mL). The neck of each flask was sealed with a syringe cap, and the flasks were flushed with nitrogen for 15 min while rocking at room temperature. Substrate in ethanol (0.18 mL) to give a final concentration of 200 μM was injected into each flask. At this point, the control flasks were vented to air, and anaerobic flasks were fitted with a nitrogen-containing balloon. All flasks were transferred to a 37

°C water bath and shaken for 60 min. Flasks were placed on ice and steroids isolated as described previously.

Assay for Time-Dependent Inactivation of Aromatase by **1c**, **2c**, **2e**, *and* **3c**. Time-dependent inactivation studies were done with primary incubations (1.0 mL) which contained buffer, microsomes (3.0 mg of protein/mL), and NADPH (10 mM). At various times after the addition of steroid (in 0.02 mL of ethanol), aliquots (0.02 mL) were removed and added to assay tubes containing [1,2-^{3}H]androst-4-ene-3,17-dione (0.5 nmol), buffer (0.48 mL), and 1 drop of propylene glycol. After 5 min, assay reactions were terminated by the addition of chloroform (5 mL) and by vortexing for 35 s. The remaining aromatase activity was calculated from ^3H_2O production as described previously (Covey et al., 1981).

In an alternate protocol, primary incubations (as described above, except scaled up to 6.0 mL) were assayed prior to addition of steroid (1.2 μmol in 0.12 mL of ethanol) and following 35 min of incubation at 37 °C with steroid. Microsomes were pelleted (0° C, 60 min, 100000*g*) and resuspended in fresh buffer to a protein concentration of ≈3 mg/mL. Aliquots were taken for protein and activity assays.

Incubation of **2c** *and* **2e** *with Placental Microsomes and* $^{18}O_2$. Microsomes (27 mg of protein), NADPH (10 mM), and buffer (total volume 8.82 mL) were placed in 50-mL two-necked round-bottom flasks. One neck was fitted with a syringe cap and the other with a vacuum adapter. Flasks were flushed with N_2 for 10 min while shaking at 37 °C and subsequently evacuated before being filled with $^{16}O_2$ or $^{18}O_2$. Substrate (1.8 μmol in 0.18 mL of ethanol) was injected into each flask prior to shaking in a 37 °C bath for 60 min. Flasks were placed on ice and steroids isolated as described previously. One-fifth of the isolated steroids was acetylated and analyzed by GC to determine per cent conversion to **3c**; the remainder was analyzed by SIM-GC–MS to determine ^{18}O content of the product **3c**.

Incubation of **3c** *with Microsomes and* $H_2{}^{18}O$. Microsomes (3 mg of protein) and buffer in a total volume of 980 μL were lyophilized. A control experiment established that no loss of aromatase activity occurred during lyophilization. The resulting powder was taken up in 980 μL of $H_2{}^{18}O$ and supplemented with NADPH (10 mM) and **3c** (0.2 μmol in 20 μL of ethanol). Incubation and recovery of steroids were done as described earlier. Steroids were analyzed by GC and SIM-GC–MS to determine ^{18}O content of the recovered **3c**.

Gas Chromatographic Methods. Steroids analyzed as their acetate derivatives were prepared as follows: 0.1 mL each of acetic anhydride and pyridine was added to approximately 1 μmol of steroid, and the mixture was heated at 50 °C for 4 h. The reaction was stopped by the addition of 1 mL of water and 1 mL of methylene chloride. The water layer was removed, and the organic layer was extracted with 5% HCl until the aqueous layer remained acidic. The organic layer was then extracted once with 5% $NaHCO_3$ and dried over Na_2SO_4.

GC analyses were performed on a Varian 3700 (N_2, 40 mL/min; injector, 280 °C; detector, 280 °C) with a 6 ft × 2 mm i.d. glass column containing 1% SP2401 on 80/100-mesh Gaschrom Q. Program conditions were as follows: initial temperature, 190 °C; final temperature, 250 °C; rate, 6 deg/min.

Mass Spectrometric Analyses. All analyses were performed on a Finnigan 3200 GC–MS (He, 30 mL/min; separator, 240 °C; injector, 220 °C; ionizing energy, 70 eV) with a 2 ft × 4 mm i.d. glass column containing 1% SP2401 on 80/100-mesh Gaschrom Q. The GC program given earlier was used for scanning electron impact studies. SIM studies of molecular ions were done isothermally at 240 °C.

Table I: Metabolites Isolated from Incubation of Placental Microsomes with 19-Methyl-Substituted Steroids

substrate	product	R_f[a]	retention time (min)[b]	molecular ion
1c		0.79	9.0	300
	17β-hydroxy-10-ethylestr-4-en-3-one	0.63	9.0	344[c]
2c		0.33	12.0	358[c]
	17β-hydroxy-10-[(1*S*)-1-hydroxyethyl]estr-4-en-3-one	0.18	12.1	402[d]
	3c	0.57	10.1	314
	10-acetyl-17β-hydroxyestr-4-en-3-one	0.33	10.4	358[c]
2e		0.36	12.5	358[c]
	17β-hydroxy-10-[(1*R*)-1-hydroxyethyl]estr-4-en-3-one	0.23	12.5	402[d]
	3c	0.58	10.1	314
	10-acetyl-17β-hydroxyestr-4-en-3-one	0.36	10.3	358[c]
3c		0.59	10.1	314
	10-acetyl-17β-hydroxyestr-4-en-3-one	0.38	10.4	358[c]

[a] Estrone R_f 0.73; estradiol R_f 0.66; compound **8** R_f 0.53. [b] The acetate derivatives of estrone and estradiol have GC retention times of 6.9 and 7.2 min, respectively. The acetate derivative of **7** had a GC retention time of 14.5 min, while **8** had a retention time of 10.0 min. [c] Analyzed as monoacetate. [d] Analyzed as diacetate.

Difference Spectroscopy. Difference spectra were recorded on a Cary 118 spectrophotometer with a scattered transmission accessory. All spectra were recorded at room temperature with a slit width of 0.2 mm and a full-scale absorbance of 0.02 AU. Reference and sample cuvettes (3 mL) contained microsomes suspended in incubation buffer (1.2 mg of protein/mL) and were scanned from 470 to 360 nm. Equal volumes of ethanol or steroid in ethanol were added to the reference and sample cuvette, respectively; total addition to each cuvette did not exceed 15 μL. Ethanol itself did not induce a spectral change in the microsomes. Spectral binding constants were obtained from a linear regression analysis of a plot of ΔA^{-1} versus (steroid concentration)$^{-1}$. P-450 content of microsomes was determined by the method of Estabrook et al. (1972) with a millimolar extinction coefficient of 100 for $A_{450} - A_{510}$.

RESULTS

Microsomal Metabolism of **1c**, **2c**, **2e**, *and* **3c** *in the Presence of NADPH*. The results of these studies are summarized in Table I. None of the analytical methods employed could detect estrone or estradiol production from the four steroids studied. A control experiment established that as low as 5% conversion of starting material to estrogens could be detected by GC. A 17β-HSD product was generated from all four steroids. For **1c** and **3c**, these 17β-hydroxy steroids were the only metabolites found. No product corresponding to the anomalous oxygenation product **7** was found in incubations of **1c**, either as its acetate or as the 5,10 cyclic ether **8**. Both **2c** and **2e** were converted to a product whose chromatographic mobility and mass spectrum were identical with those of **3c**. Another product isolated from incubations of **2c** and **2e** was identical with the 10-acetyl-17β-hydroxyestr-4-en-3-one isolated from incubations of **3c**.

Competitive Inhibition of Aromatase. The four 19-methyl-substituted steroids all acted as competitive inhibitors of aromatase. The affinity of **1c** (K_I = 81 ± 32 nM) for aromatase was comparable to that of androstenedione (K_m = 91 nM). Steroid **1c** has previously been reported to have an

I_{50} of 300 nM (Marcotte & Robinson, 1982). The affinities of **2c** and **2e** for the enzyme were similar to one another (K_I = 11.0 ± 4.9 μM and 9.9 ± 2.4 μM, respectively) but ≈50-fold less than that of 19-hydroxyandrostenedione (K_I = 210 nM; Kelly et al., 1977), the normal intermediate to which they are analogous. The affinity of 19-oxoandrostenedione (K_I = 260 nM; Kelly et al., 1977) has been reported to be similar to that of 19-hydroxyandrostenedione, but in the 19-methyl series of analogues, **3c** (K_I = 150 ± 13 nM) bound ≈65 times more tightly to the enzyme than either **2c** or **2e**.

Difference Spectroscopy of 19-Methyl-Substituted Steroids. Each of the steroids **1c**, **2c**, **2e**, and **3c** induced a type I difference spectrum in placental microsomes, with an absorption maximum at 386 nm and a minimum at 418 nm. The magnitude of the spectral change induced by **1c**, **2e** and **3c** [(1.74 ± 0.05), (1.59 ± 0.10), and (1.62 ± 0.04) × 10^{-4} AU per nmol of P-450, respectively] was similar to that induced by androstenedione (1.65 × 10^{-4} AU per nmol of P-450). In contrast, **2c** consistently exhibited less of a spectral change, approximately 60% of that seen with the other steroids [(1.18 ± 0.11) × 10^{-4} AU per nmol of P-450)].

In nonsolubilized microsomes, the K_s of androstenedione, **1c**, and **3c** agreed with the corresponding K_m and K_I values (K_s = 41 nM, 100 ± 36 nM, and 75 ± 13 nM vs K_m or K_I = 91 nM, 81 nM, and 150 nM, respectively). However, alcohols **2c** and **2e** had K_s values (600 ± 160 nM, 140 ± 32 nM) substantially lower than their K_I values (11 μM, 9.9 μM). The possibility that **2c** and **2e** were preferentially partitioning into the microsomes resulting in an anomalously low K_s prompted us to solubilize placental microsomes by a protocol provided by Dr. L. E. Vickery.[3] The K_s of **2c** and **2e** determined in solubilized microsomes (2.3 μM and 1.3 μM, respectively) was greater than the K_s determined in nonsolubilized microsomes but was still 5–10 times less than K_I values of these steroids. Solubilization of microsomes had little effect on the K_s of androstenedione (41 nM vs 89 nM) or steroid **1c** (100 nM vs 71 nM) but did increase the K_s of **3c** by 10-fold (75 nM vs 780 nM). Previously, the K_s of androstenedione in solubilized placental microsomes has been reported as 13 nM (Zachariah et al., 1976).

In additional experiments using solubilized microsomes, 5 μM miconazole elicited a type II_L difference spectrum as has been reported previously (Kellis & Vickery, 1984; Mason et al., 1985). In contrast, 5 μM miconazole elicited a type II_H difference spectrum from solubilized microsomes containing steroid (1 μM androstenedione, **1c**, and **3c**; 10 μM **2c** and **2e**). These results are consistent with binding of steroids **1c**, **2c**, **2e**, and **3c** to the active site of aromatase.

Inactivation of aromatase in nonsolubilized placental microsomes by pretreatment with 4-OHA abolished the reduced CO difference spectrum as well as the type I binding spectrum normally induced by steroids **2c** and **2e**. Parallel controls subjected to the same treatment but lacking 4-OHA retained these spectral properties.

Cofactor Requirements for the Microsomal Metabolism of **2c** *and* **2e**. A requirement for O_2 and reduced pyridine nucleotide cofactor in the microsomal conversion of **2c** and **2e** to **3c** was established by varying the cofactors supplied to incubations. Total steroids isolated from each incubation were analyzed by TLC, acetylated without fractionation, and analyzed by GC.

In one set of experiments, alcohols **2c** and **2e** were incubated with microsomes in the presence of NAD, NADH, NADP, or NADPH while open to atmosphere. By TLC, **3c** and the 17β-HSD metabolites of **2c** and **2e** could be observed only in incubations containing NADH or NADPH. By GC, incubations of **2c** and **2e** with NAD or NADP did not produce any detectable **3c** or 17β-hydroxy-10-acetylestr-4-en-3-one, while NADH and NADPH proved equally effective as cofactors in the oxygenation of **2c** and **2e**. NADH has previously been shown to be a competent cofactor for aromatase (Sheean & Meigs, 1983). The conversion of **2e** to **3c** (59.2 ± 1.2%) was ≈1.75 times greater than for **2c** (33.6 ± 5%) in the presence of either NADH or NADPH.

In another set of experiments, **2c** and **2e** were incubated with microsomes and NADPH either under N_2 or open to the atmosphere. A separate control experiment established the inhibition of 19-hydroxyandrostenedione aromatization under N_2. By TLC the 17β-HSD product of each substrate was generated in both the presence and absence of oxygen. Production of **3c** was detectable by TLC and GC only in incubations open to the atmosphere. Again, conversion of **2e** to **3c** (63.6%) was greater than for **2c** (43.7%).

Table II: Effect of Aromatase Inactivation on Metabolism of **2c** and **2e** by Placental Microsomes

substrate	preincubation	percent conversion to **3c**[a] expt 1	percent conversion to **3c**[a] expt 2	specific activity of microsomes[b] expt 1	specific activity of microsomes[b] expt 2
2c	−4-OHA	32.1	30.7	196	179
	+4-OHA	ND[c]	ND	2.5	11.9
2e	−4-OHA	45.3	46.9	205	168
	+4-OHA	ND	ND	1.3	4.5

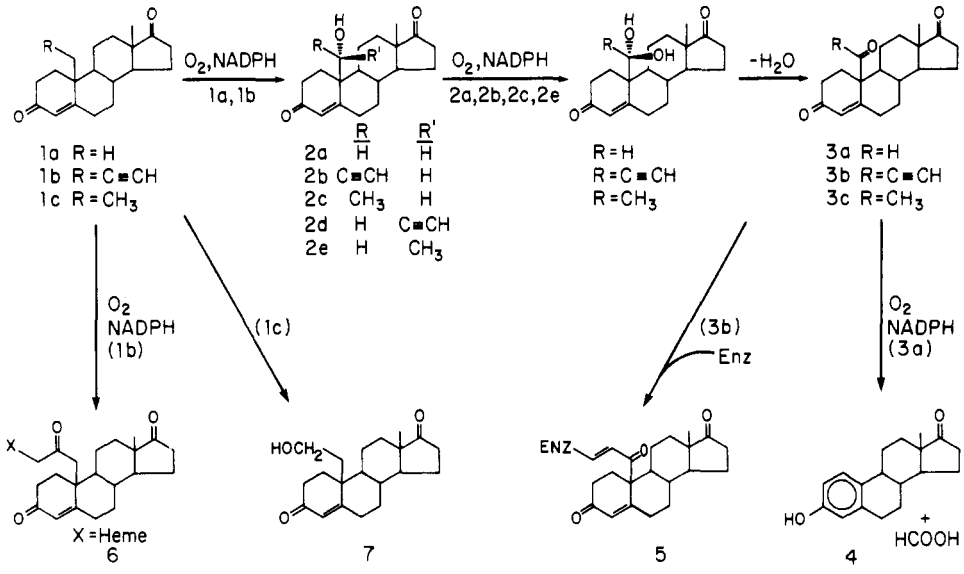
[a] Determined by GC analysis of total recovered steroids after acetylation. [b] Determined after preincubation, pelleting, and resuspension; expressed as pmol of estrogen min^{-1} (mg of protein)$^{-1}$. Microsomes in experiments 1 and 2 had initial specific activities of 248 and 226 pmol of estrogen min^{-1} (mg of protein)$^{-1}$, respectively. [c] ND, none detectable.

Effect of Aromatase Inactivation on the Metabolism of **2c** *and* **2e** *by Placental Microsomes.* In order to establish that aromatase was responsible for the conversion of **2c** and **2e** to **3c**, microsomes were preincubated with NADPH and 4-OHA, a suicide inactivator of aromatase (Brodie et al., 1981; Covey & Hood, 1982). After pelleting and resuspending, these microsomes were incubated with **2c** or **2e** in the presence of NADPH. Microsomes in control incubations went through the same treatment but were not exposed to 4-OHA. Steroids recovered from these incubations were analyzed by TLC and GC. The results of these studies are summarized in Table II. Preincubation of microsomes with 4-OHA resulted in >90% loss of aromatase activity whereas controls not exposed to 4-OHA lost <25% of their starting activity. By TLC, the 17β-HSD products of **2c** and **2e** were observable in aromatase-inactivated as well as control incubations. By both TLC and GC, **3c** and its 17β-HSD product were observable only in control incubations.

Aromatase Inactivation by Steroids **2c**, **2e**, *and* **3c**. As reported previously (Marcotte & Robinson, 1982), steroid **1c** did not inactivate aromatase. A similar experiment with **2e** proved inconclusive in that some slight inactivation of aromatase (≈25% loss of activity relative to control at 20 μM) occurred during a 35-min preincubation. This loss of activity may have been attributable to the generation of a high-affinity metabolite (**3c**) that competitively inhibited aromatization during the assay step. For this reason, microsomes were subjected to 35-min preincubation with the steroid and NADPH and then pelleted, resuspended, and assayed for remaining activity. Such experiments with steroids **2c** and **2e** (200 μM) revealed a 20% loss in aromatase activity relative to controls that were preincubated without steroid. Preincu-

[3] L. E. Vickery, unpublished results.

Scheme I



bation of microsomes with **3c** (200 μM) in the presence of NADPH resulted in recovery of only 65 ± 8% of initial activity. Corresponding controls resulted in complete recovery of initial activity (preincubation = no steroid plus 10 mM NADPH, 102 ± 12% recovery; preincubation = 200 μM 19-oxoandrostenedione plus 10 mM NADPH, 97 ± 11% recovery; preincubation = 200 μM **3c** minus NADPH, 102 ± 11% recovery). Thus, the inactivation of aromatase by **2c** and **2e** at high concentrations may occur after they have been converted to **3c**.

Fate of Oxygen in the Conversion of **2c** *and* **2e** *to* **3c**. Previous work has shown that oxygen used in the second of aromatase's three monooxygenations (**2a** → **3a**, see Scheme I) is not retained in the final product, formic acid (Akhtar et al., 1982). To determine if oxygen used in the analogous conversion of **2c** and **2e** to **3c** was retained, both alcohols were incubated with microsomes in the presence of NADPH and $^{18}O_2$. Percent conversion of each substrate to **3c** was determined by GC, and the ^{18}O content of **3c** was determined by GC–MS-SIM of the molecular ion region. Such incubations of **2c** yielded **3c** containing very little ^{18}O (2.8 and 2.7%; 26.2 and 28.6% conversion of **2c** to **3c**) while **3c** arising from **2e** retained a substantial amount of ^{18}O (71.2 and 68.9%; 37.2 and 41.1% conversion of **2e** to **3c**). In a separate control experiment, **3c** was incubated with microsomes and NADPH in $H_2{}^{18}O$. The recovered **3c** contained less than 4% ^{18}O, suggesting that exchange of ^{18}O out of **3c** generated from **2c** and **2e** would be minimal.

Discussion

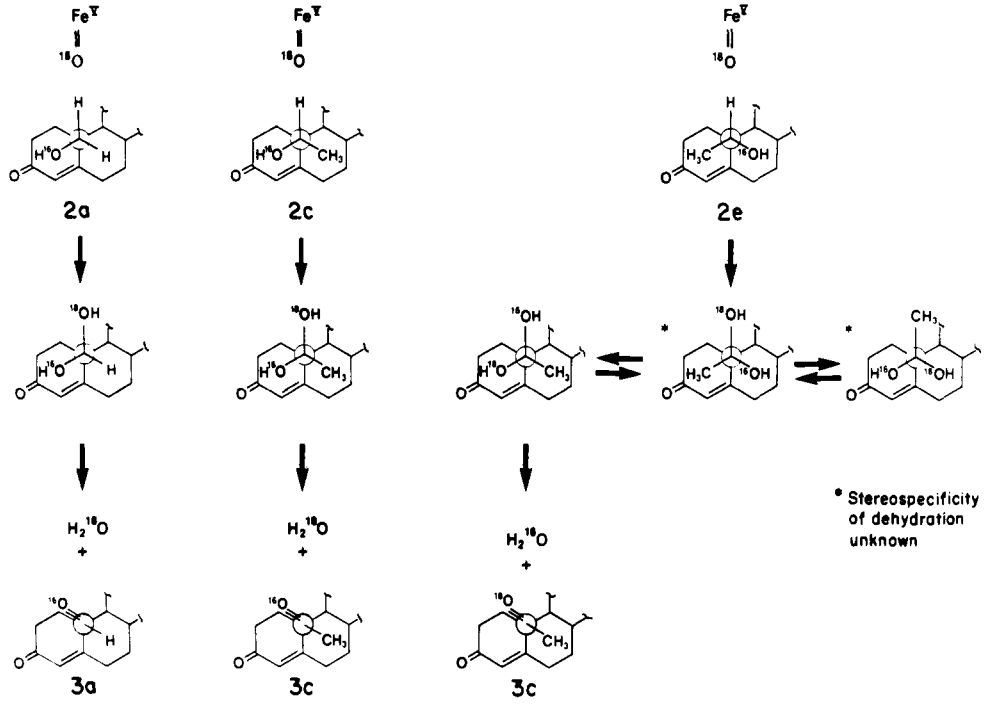
The goal of this study was to determine if the presence of a substrate 19-alkyl group could perturb the regiospecificity of aromatase oxygenation. The methods employed did not detect products indicative of a change in the enzyme's regiospecificity when presented with the substrate/intermediate analogues **1c**, **2c**, **2e**, and **3c**. Instead, the presence of a 19-methyl substituent blocked the microsomal oxygenation of compounds **1c** and **3c** as evidenced by the failure to detect their conversion to oxygenated metabolites. In contrast, both intermediate analogues for the second oxygenation of aromatase (**2c** and **2e**) were converted to **3c**, the 19-methyl congener of the normal product of this oxygenation, 19-oxoandrostenedione.

It is unclear why the oxygenation of **1c** but not that of **2c** and **2e** should be blocked in these 19-methyl analogues, especially since both androstenedione and 19-hydroxyandrostenedione are believed to undergo oxygenation in the same aromatase active site (Kelly et al., 1977). Compound **1c** binds to the active site of aromatase as evidenced by its ability to competitvely inhibit androstenedione aromatization and to induce a type I difference spectrum in placental microsomes, but it is not oxygenated. Perhaps compound **1c** is bound to the cytochrome with the 19-methyl group so close to the iron atom that it interferes with the productive binding of oxygen to this complex. If so, then the 19-methyl group of **1c** must be oriented differently than the 19-methyl group of compounds **2c** and **2e** when bound to aromatase. Otherwise, none of the compounds would be monooxygenated. Presumably, active site accommodation of the additional hydroxyl group in compounds **2c** and **2e** is responsible for their altered mode of binding.

The inability of NAD and NADP to support the conversion of **2c** and **2e** to **3c** makes the involvement of a 19-dehydrogenase in this transformation unlikely, especially since incubation of **3c** with NADPH and microsomes did not result in the generation of 19-hydroxy metabolites. The cofactor requirements (NADPH and O_2) for conversion of **2c** and **2e** to **3c** are consistent with the involvement of a P-450. Spectroscopically observable P-450 in placental microsomes is thought to be associated primarily with aromatization (Zachariah, 1976). Since compounds **2c** and **2e** both elicit the type I difference spectrum indicative of a P-450/steroid complex (Jefcoate, 1978), a strong case for their binding to microsomal aromatase can be made. The competitive inhibition of androstenedione aromatization by both compounds also indicates that they bind to microsomal aromatase. Although compelling evidence for the binding of **2c** and **2e** to aromatase has been presented, it is not possible with a microsomal preparation to prove unequivocally that aromatase and not a previously undescribed P-450 is responsible for the oxidation of **2c** and **2e** to **3c**. We attribute the oxidation of these compounds to aromatase because the aromatase suicide inactivator, 4-OHA, blocks the conversion of both compounds to **3c** and destroys the type I binding spectrum elicited by compounds **2c** and **2e**.

The oxidation of steroidal alcohols to ketones by P-450 enzymes is not without precedent. The 17α-hydroxylase/17–20 lyase (P-450 SCCII) purified from pig testis has been shown to oxidize testosterone to androstenedione (Suhara et al., 1984). It would be interesting to know if 4-androsten-3-one

Scheme II



could be 17α-hydroxylated by P-450 SCCII. If not, there would be a similarity between the oxidative capability of this P-450 and aromatase's ability to oxidize compounds **2c** and **2e** while failing to oxygenate compound **1c**.

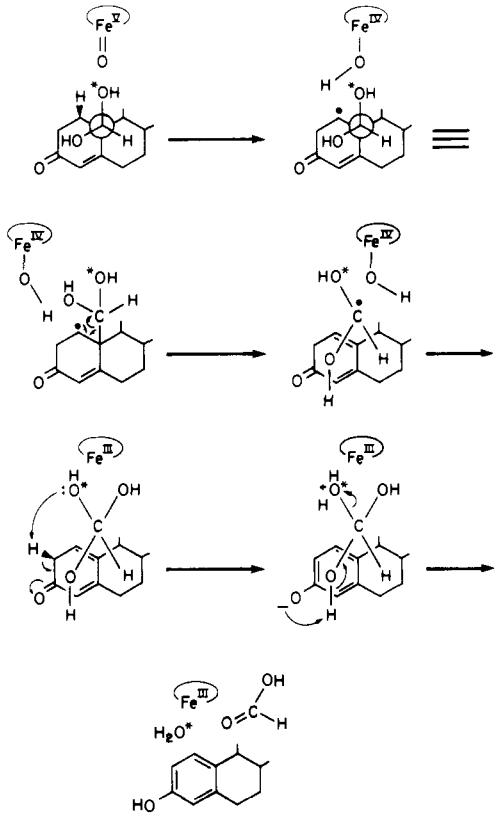
The conversion of 19-hydroxyandrostenedione (**2a**) to 19-oxoandrostenedione (**3a**) (see Scheme I) proceeds with stereospecific insertion of oxygen into the 19-*pro-R* carbon–hydrogen bond of **2a** (Skinner & Akhtar, 1969; Arigoni et al., 1975; Osawa et al., 1975). Therefore, it was anticipated that only **2c** would be metabolized, since the C_{19}–hydrogen bond normally oxygenated is not replaced by a methyl group as it is in **2e**. Thus, the metabolism of both diastereomeric alcohols **2c** and **2e** attributed to aromatase is noteworthy. As shown in Scheme II (top row), the results could suggest that the two diastereomers bind to aromatase with differing orientations about the C_{10}–C_{19} bond. Since oxygen delivery to the substrate is believed to occur from the out-of-ring position (Osawa, 1973; Duax & Osawa, 1980), **2c** could bind to the active site with the 19-hydroxyl positioned over ring A, just as it would be for **2a**. In order to present a C–H bond to the enzyme for oxygenation, **2e** would have to bind in a conformation having the 19-hydroxyl over ring B. Since conversion of **2e** to **3c** is always greater than the conversion of **2c** to **3c** (by 50–75%), it seems that the enzyme is stereoselective with respect to these substrates, although the selectivity (*R* rather than *S*) does not reflect aromatase's preference for removal of the 19-*pro-R* hydrogen of 19-hydroxyandrostenedione. An alternate explanation for the metabolism of both **2c** and **2e** by aromatase may be that the two steroids are oxygenated by different mechanisms. While **2c** could undergo the normal hydrogen abstraction at C_{19}, **2e** may actually undergo hydrogen abstraction from the 19-methyl group. Rearrangement (via hydrogen migration) of the resulting primary radical to a more stable C_{19} secondary radical which then participated in oxygen rebound would result in the product **3c**. The methods employed in this study cannot eliminate this possibility.

Previous studies have shown that oxygen used in the second of aromatase's three monooxygenations is not retained in the product, formic acid (Akhtar et al., 1982). Since **2c** and **2e** are analogues of the substrate for the second oxygenation (19-hydroxyandrostenedione), we sought to determine if $^{18}O_2$ used in the metabolism of **2c** and **2e** would also be retained in the product **3e**. For the *S* isomer **2c**, our finding of very little ^{18}O retention is explainable as shown in Scheme II. Following oxygenation, the *gem*-diol of **3c** would be subject to the same enzyme–substrate interactions that dictate stereospecific elimination of the newly formed hydroxy group in the *gem*-diol of 19-oxoandrostenedione (**3a**). In contrast, the *R* isomer **2e** retained an amount of ^{18}O ($\approx$70%) during its metabolism to **3c** that was midway between the amount expected from random dehydration of the *gem*-diol initially formed (50%) and that expected from stereospecific dehydration (100%). As shown in Scheme II, the *gem*-diol produced from **2e** could undergo dehydration (of unknown stereospecificity) or prior to dehydration rotate to one of the two other conformers shown. In one of these conformers (as discussed above), the enzyme mediates stereospecific dehydration of the *gem*-diol, while the stereospecificity of dehydration from the other is unknown. Thus the retention of ^{18}O in **3c** produced from **2e** may reflect dehydrations from three different enzyme-bound conformers, only one of which may lead to a stereospecific result.

An explanation for the failure of **3c** to be metabolized by aromatase could lie in the hydration state of its 19-ketone. Oxygenation of 19-oxoandrostenedione may require that the hydrated form of this aldehyde (the *gem*-diol) be present. This has been proposed previously (Osawa & Shibata, 1974) to explain the results of isotopic labeling studies. As a ketone, **3c** is less likely to undergo hydration to its *gem*-diol form than is 19-oxoandrostenedione. Thus, **3c** may bind to the enzyme but be unable to form the *gem*-diol required for oxygenation to estrone. If generated in the active site from **2c** or **2e** as the *gem*-diol, **3c** may dehydrate faster than the third oxygenation can occur. Thus, metabolism of **2c** and **2e** would stop after the second oxygenation, and **3c** rather than estrone would be released by the enzyme.

Because the hypothetical intermediacy of 19,19-dihydroxyandrostenedione (Osawa & Shibata, 1974) provides a reasonable basis for understanding why compounds **2c**, **2e**, and **3c** were not aromatized, we considered its aromatization

Scheme III



in detail to ascertain that all of the data relevant to the third aromatase monooxygenation could be accommodated. As will be discussed shortly, there are differences between the mechanism shown in Scheme III and the earlier mechanism suggested by Osawa and Shibata (1975, 1977). We propose (Scheme III) that the third monooxygenation is initiated by hydrogen abstraction at C_1, followed by homolytic cleavage of the C_{10}–C_{19} bond to generate a 19-*gem*-diol carbon radical (plus the $\Delta^{1(10),4}$-3-ketosteroid), and terminated by oxygen rebound occurring at this carbon radical to generate orthoformate. Since electron-withdrawing groups on carbon atoms are known to facilitate carbon radical formation (Hine, 1962), it is reasonable that homolytic cleavage of the C_{10}–C_{19} bond would be enhanced by the dihydroxy groups of C_{19}. Incipient aromatization of the $\Delta^{1(10),4}$-3-ketosteroid also makes the proposed homolytic cleavage reaction energetically favorable and irreversible.

Orthoformate has been proposed previously (Osawa & Shibata, 1975, 1977) as the product that results from the final aromatase monooxygenation. The mechanism suggested for its formation involved dioxygen and heterolytic cleavage of the C_{10}–C_{19} bond in a cyclic transition state. The mechanism shown in Scheme III differs from that of Osawa and Shibata in that homolytic cleavage of the C_{10}–C_{19} bond and perferryl oxygen (Fe^{V}=O) in noncyclic transition states are proposed. Groves et al. (1981) have obtained evidence from model systems which suggests that the active oxygen species might be better represented as one of its resonance structures (the Fe^{IV} porphyrin radical cation) but formal perferryl oxygen is used here for convenience. The mechanistic details of perferryl oxygen reduction during the catalytic reactions shown in Scheme III are consistent with those discussed in reviews on the catalytic mechanism of cytochrome P-450 reactions (Dolphin, 1985; Guengerich & MacDonald, 1984).

The newly formed orthoformate, sandwiched in the enzyme active site between the heme group and the steroid (and possibly hydrogen bonded to both), seems ideally located for the proton-transfer and dehydration reactions shown in the remainder of Scheme III. The hydroxyl group in the orthoformate involved in 2β-proton abstraction is derived from the second monooxygenation reaction, while the one deprotonated by the steroid phenoxide group is derived from the first monooxygenation reaction. Thus, when dehydration of the orthoformate occurs, the *protonated* hydroxyl group from the second monooxygenation will leave as water while the oxygen atoms from the first and third monooxygenations will remain in the formic acid. Alternative dehydration schemes (not shown) in which an enzyme nucleophile deprotonates either of the *unprotonated* hydroxyl groups would also lead to the desired outcome and are equally plausible. Product release is postulated to occur *after* stereospecific dehydration of the orthoformate. The entire mechanistic sequence shown in Scheme III accounts for the stereospecific loss of the 1β- and 2β-hydrogens and the incorporation of the first and third oxygen atoms into formic acid as required by previous studies (Townsley & Brodie, 1968; Brodie et al., 1969; Akhtar et al., 1982). Thus, the mechanism proposed in Scheme III is consistent with what is known about the third aromatase monooxygenation and can rationalize the results obtained with compounds **2c**, **2e**, and **3c**.

Acknowledgments

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Supplementary Material Available

Table I listing atomic coordinates and Table II listing anisotropic vibration parameters for 10-[(1*R*)-1-hydroxyethyl]estr-4-ene-3,17-dione (2 pages); Table III listing calculated structure factors for 10-[(1*R*)-1-hydroxyethyl]estr-4-ene-3,17-dione (11 pages). Ordering information is given on any current masthead page.

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