THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 291, NO. 33, pp. 17143–17164, August 12, 2016

Mechanism of 17α,20-Lyase and New Hydroxylation Reactions of Human Cytochrome P450 17A1

^{18}O LABELING AND OXYGEN SURROGATE EVIDENCE FOR A ROLE OF A PERFERRYL OXYGEN*[S]

Received for publication, April 15, 2016, and in revised form, May 31, 2016 Published, JBC Papers in Press, June 23, 2016, DOI 10.1074/jbc.M116.732966

Francis K. Yoshimoto[‡§1], **Eric Gonzalez**[‡1], **Richard J. Auchus**[§], **and F. Peter Guengerich**[‡2]

From the [‡]*Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146 and the* [§]*Division of Metabolism, Diabetes and Endocrinology, Departments of Internal Medicine and Pharmacology, University of Michigan, Ann Arbor, Michigan 48019*

Cytochrome P450 (P450) reactions can involve C–C bond cleavage, and several of these are critical in steroid and sterol biosynthesis. The mechanisms of P450s 11A1, 17A1, 19A1, and 51A1 have been controversial, in the context of the role of ferric peroxide (FeO_2^-) *versus* perferryl (FeO^{3+}, compound I) chemistry. We reinvestigated the 17α-hydroxyprogesterone and 17α-hydroxypregnenolone 17α,20-lyase reactions of human P450 17A1 and found incorporation of one ^{18}O atom (from $^{18}O_2$) into acetic acid, consonant with proposals for a ferric peroxide mechanism (Akhtar, M., Lee-Robichaud, P., Akhtar, M. E., and Wright, J. N. (1997) *J. Steroid Biochem. Mol. Biol.* 61, 127–132; Akhtar, M., Wright, J. N., and Lee-Robichaud, P. (2011) *J. Steroid Biochem. Mol. Biol.* 125, 2–12). However, the reactions were supported by iodosylbenzene (a precursor of the FeO^{3+} species) but not by H_2O_2. We propose three mechanisms that can involve the FeO^{3+} entity and that explain the ^{18}O label in the acetic acid, two involving the intermediacy of an acetyl radical and one a steroid 17,20-dioxetane. P450 17A1 was found to perform 16-hydroxylation reactions on its 17α-hydroxylated products to yield 16,17α-dihydroxypregnenolone and progesterone, suggesting the presence of an active perferryloxo active species of P450 17A1 when its lyase substrate is bound. The 6β-hydroxylation of 16α,17α-dihydroxyprogesterone and the oxidation of both 16α,17α-dihydroxyprogesterone and 16α,17α-dihydroxypregnenolone to 16-hydroxy lyase products were also observed. We provide evidence for the contribution of a compound I mechanism, although contribution of a ferric peroxide pathway in the 17α,20-lyase reaction cannot be excluded.

Cytochrome P450 (P450)[3] enzymes catalyze oxidations of more chemicals than any other group of proteins (1). The list of reactions includes aliphatic and aromatic hydroxylations, heteroatom oxidations, epoxidations, and reactions involving both ring formation and cleavage (2–4). Many P450 reactions are important in the biosynthesis and degradation of steroids and sterols (4, 5), including several critical C–C bond cleavage reactions, *i.e.* those catalyzed by P450s 11A1, 17A1 (Fig. 1), 19A1, and 51A1 (6, 7).

The mechanisms of the C–C cleavage reactions have been the subject of considerable interest and debate. One of the questions with P450s 17A1, 19A1, and 51A1 has been whether the active oxidant is a ferric peroxide (FeO_2^-), which is an early intermediate following oxygen addition to the iron (Fig. 2, *step 4*) or the FeO^{3+} species (Fig. 2, *step 6*), often referred to as compound I (4, 10, 11). With P450s 17A1 and 19A1, a variety of approaches has been applied, including theoretical calculations, biomimetic models, spectroscopy, substrate atom labeling, and kinetics (12–32).

These C–C bond cleavage reactions are complex, and many of the results are ambiguous; also, a "mixed" mechanism would not be discerned in many of these experiments. One powerful approach originally used by Akhtar and co-workers (27–31) analyzes the actual reaction and can provide discrimination between the nucleophilic FeO_2^- and electrophilic FeO^{3+} reactions (Fig. 2), based on the incorporation of ^{18}O label from O_2 into the carboxylic acid products (Fig. 3) (7). However, these experiments are complicated due to the ubiquitous presence of formic acid (P450 19A1 and 51A1 reactions) and acetic acid (P450 17A1) in laboratory settings. Thus, the data from such experiments are interpreted with the most confidence when the steroid substrates are labeled with ^2H or ^{13}C isotopes to facilitate analysis (15, 33). Even then, the mass spectrometry results can be problematic, particularly if a shift of only one atomic mass unit is introduced and isotopologues derived from ^{18}O incorporation are not discriminated from molecules containing natural abundance ^{13}C atoms (33).

The incorporation of one atom of ^{18}O label from O_2 into formic acid (Fig. 3*A*) had been considered one of the most critical pieces of evidence in support of an FeO_2^- mechanism for P450 19A1 (14, 15, 34). Because of the importance of this evidence in the existing dogma, we re-examined this experiment using several technical improvements including the following: (i) purified recombinant P450 19A1; (ii) a new diazo reagent

* This work was supported, in whole or in part, by National Institutes of Health Grants R37 CA090426, R01 GM118122, and R01 GM103937 (to F. P. G.) and R01 GM086596 (to R. J. A.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

[S] This article contains supplemental Figs. S1–S4.

[1] Both authors contributed equally to this work.

[2] To whom correspondence should be addressed: Dept. of Biochemistry, Vanderbilt University School of Medicine, 638B Robinson Research Bldg., 2200 Pierce Ave., Nashville, TN 37232-0146. Tel.: 615-322-2261; Fax: 615-343-0704; E-mail: f.guengerich@vanderbilt.edu.

[3] The abbreviations used are: P450, cytochrome P450; compound I, formal FeO^{3+} form of a hemoprotein; HMBC, homonuclear correlation (NMR) spectroscopy; DHEA, dehydroepiandrosterone; ESI, electrospray ionization; HRMS, high resolution mass spectrometry; LC-MS, (combined) liquid chromatography-mass spectrometry.

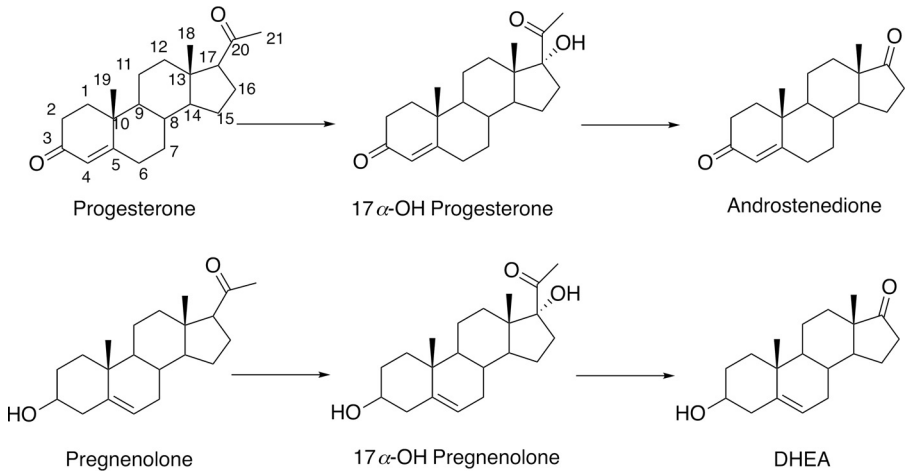


FIGURE 1. **Steroid 17α-hydroxylation and 17α,20-lyase reactions catalyzed by P450 17A1.**

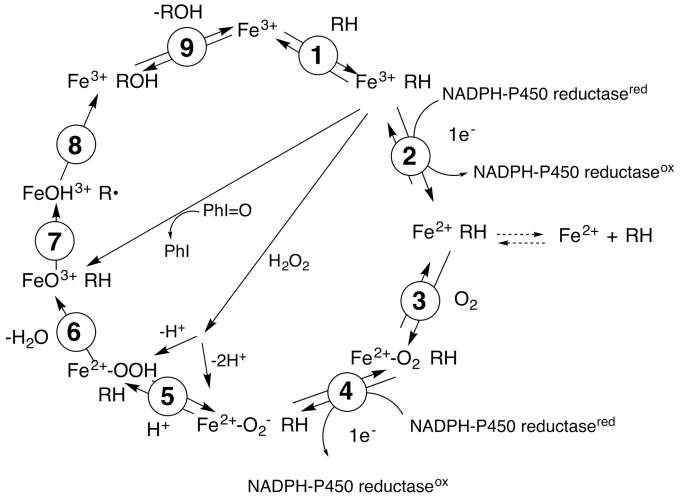


FIGURE 2. **Classic catalytic cycle of P450 enzymes (4).** Paths for oxygen surrogates (PhI=O, H_2O_2) are also included. Note the FeO_2^- (ferric peroxide) and FeO^{3+} (compound I) forms discussed in the text. In the literature there exists different nomenclature for the same iron intermediates in this P450 catalytic cycle (*i.e.* $Fe^{III}O_2^-$, $Fe^{III}O_2H$, $Fe^{IV}O^+$, and $Fe^{IV}OH$) (8, 9). For clarity throughout the text, compound I is referred to interchangeably with FeO^{3+}, and ferric peroxide is referred to interchangeably with FeO_2^-. The electron transfers from the reductase are simplifications in that the course of electron flow is probably from $FMNH_2$/$FADH^•$ to $FMNH^•$/$FADH^•$ in the first reduction (step 2) and (assuming that the reductase contributes the second electron to the P450) from $FMNH^•$/$FAD^•$ to $FMNH^•$/FAD in the second reduction step 4.

with a pyridine nitrogen to facilitate positive ionization for liquid chromatography-mass spectrometry (LC-MS); and (iii) the use of high resolution mass spectrometry (HRMS) (33). The results for P450 19A1 unambiguously ruled out ^{18}O incorporation of ^{18}O label from O_2 into formic acid by distinguishing ^2H from ^{13}C isotope composition and are only consistent with an FeO^{3+} mechanism for P450 19A1 (33).

Because of the impact of the new studies (33), we re-examined the ^{18}O experiments with P450 17A1 (27–31) with the newer methodologies. Although our ^{18}O labeling results could be interpreted as support of an FeO_2^- mechanism for human P450 17A1, at least three possible FeO^{3+} mechanisms are still consistent with the data (Fig. 3, *B*, *C*, and *F*). We also employed artificial oxygen surrogates that might distinguish among mechanisms, *i.e.* iodosylbenzene, a single oxygen atom donor, and H_2O_2. Finally, we measured kinetic solvent isotope effects for the reactions, in light of inconsistencies in the field (23, 35). Our evidence now suggests that an FeO^{3+} mechanism is likely, at least in part, for the 17α,20-lyase reaction, and we also demonstrate the ability of the enzyme to catalyze additional 6β- and 16-hydroxylation reactions.

Results

Experimental Design for ^{18}O Experiments—The P450 17A1 17α,20-lyase reaction produces DHEA from 17α-hydroxypregnenolone (Fig. 1). The product acetic acid is of particular interest in determining the mechanism of P450 17A1 catalysis (Fig. 3). To unambiguously distinguish the acetic acid formed as a product of the P450 17A1 reaction, the 17α-hydroxy substrate was d_3-labeled at position C21 because of concerns about the level of endogenous acetic acid interfering with that formed in the enzyme reaction, based on our experience with 1- and 2-C carboxylic acids (36–39). Based on possible mechanisms shown in Fig. 3, the acetic acid products of $^{18}O_2$ incubations with the 17α-hydroxy steroids are as follows: $CD_3CO^{18}OH$ (mechanisms A, B, C, and Fb), a 1:2 molar ratio of CD_3COOH and $CD_3CO^{18}OH$ (mechanism D), only CD_2HCOOH (mechanism E), or only CD_3COOH (mechanism Fa). Because of the small amounts of acetic acid produced (1:1 stoichiometry with steroid, ~ 25 μmol) during incubations, the acetic acid was converted into an ester using diazoethylpyridine to facilitate characterization. In addition to the increase in mass, the ester is designed for efficient ionization attributable to the nitrogen in the pyridine ring, *i.e.* 2-(pyridin-2-yl)ethyl acetate (33) (*m/z* 166.1, "MH^+") with one ^{16}O incorporated (d_2-labeled,"MH^+ + 2") or one ^{16}O incorporated (d_3-labeled,"MH^+ + 3"), and one ^{18}O incorporated (d_3-labeled, "MH^+ + 5"). A similar approach was used for 17α-hydroxy-[2,2,4,6,6,21,21,21-$^2H_8$]progesterone.

17α-Hydroxypregnenolone and 17α-Hydroxyprogesterone ^{18}O Experiments—One ^{18}O atom was incorporated into acetic acid without deuterium loss (Fig. 4, *B–E*) when 17α-hydroxy-[21,21,21-$^2H_3$]pregnenolone was used as the substrate, ruling out the mechanism in Fig. 3*E*.

In the case of 17α-hydroxy-[2,2,4,6,6,21,21,21-$^2H_8$] progesterone as the substrate (Fig. 5), the signal-to-noise ratio of ^{18}O-incorporated acetate was three times greater than when

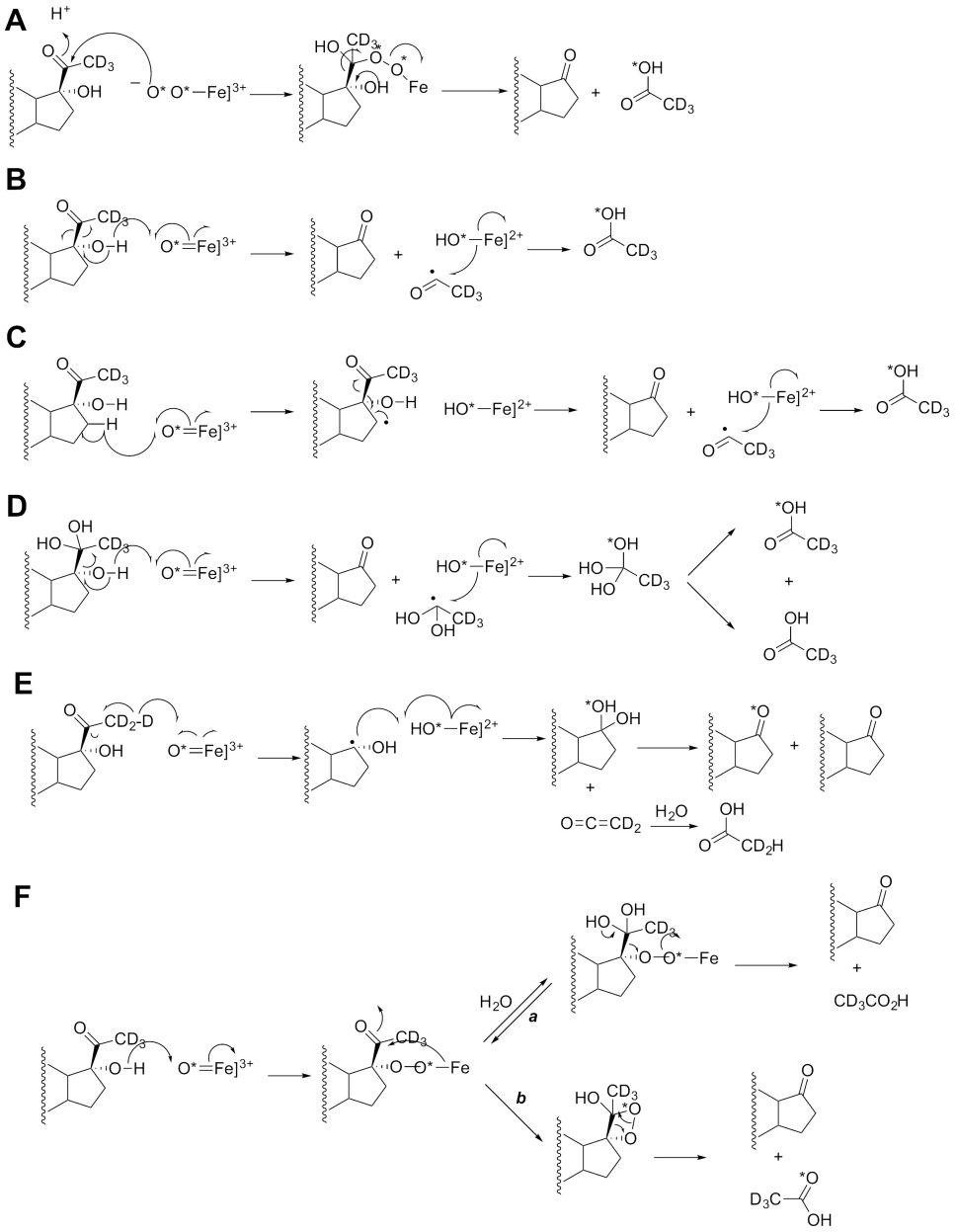


FIGURE 3. **Possible mechanisms of P450 17A1-catalyzed 17α,20-lyase reaction and expected ^{18}O labeling (7).** The course of ^{18}O (from $^{18}O_2$) and deuterium (D) labels are indicated with an *asterisk*. *A*, ferric peroxide mechanism (27–31); *B*, compound I mechanism with hydrogen atom abstraction from the 17α alcohol followed by C17-C20 bond scission to yield an acetyl radical; *C*, compound I mechanism with hydrogen atom abstraction from the C16 carbon; *D*, compound I mechanism with hydrogen atom abstraction from the 17α alcohol followed by C17-C20 bond scission to yield a hydrated acetyl radical (*gem*-diol); *E*, compound I mechanism with hydrogen atom abstraction from the C21 methyl group followed by C17-C20 bond scission to yield a C17 radical; *F*, addition of the 17α-hydroxyl group to compound I to yield an iron peroxide-C17 complex, which can decompose via either (*a*) a C20 *gem*-diol or (*b*) a C17-C20 dioxetane. See text for discussion and also Fig. 19. Mechanisms *B–D* result in an acetyl radical that undergoes oxygen rebound with Fe-*OH (compound II), with an oxygen atom from molecular oxygen ($*O_2$) into the acetic acid product.

the 17α-hydroxy-[21,21,21-$^2H_3$]pregnenolone substrate was used. This improvement in sensitivity is attributed to the extra centrifugation step to remove the emulsion when extracting the acetic acid product (*cf.* "Experimental Procedures"). Additionally, we observed a trideutero-pyridine acetate product with no ^{18}O incorporation at 6 ppm mass tolerance (Fig. 5*B*, *5b*); however, the intensity was small compared with the ^{18}O-incorporated acetate product (~0.1% of ^{18}O-incorporated product), and this isotopologue is likely derived from the residual $^{16}O_2$ in the $^{18}O_2$ cylinder (99% ^{18}O abundance).

Reactions with Oxygen Surrogates, Background and Previous Studies—If the ferric hydroperoxide mechanism is operative, then one might expect the reaction to be supported by the direct addition of H_2O_2 to ferric P450 (Fig. 2). However, Auchus and Miller (40) reported that no 17α,20-lyase activity was observed with recombinant human P450 17A1 plus H_2O_2 in yeast microsomes. Iodosylbenzene is a single oxygen donor and cannot support a reaction that requires two oxygens, *i.e.* a ferric peroxide complex (41). Iodosylbenzene also did not support the 17α,20-lyase reaction in a P450 17A1 yeast microsomal system (40).

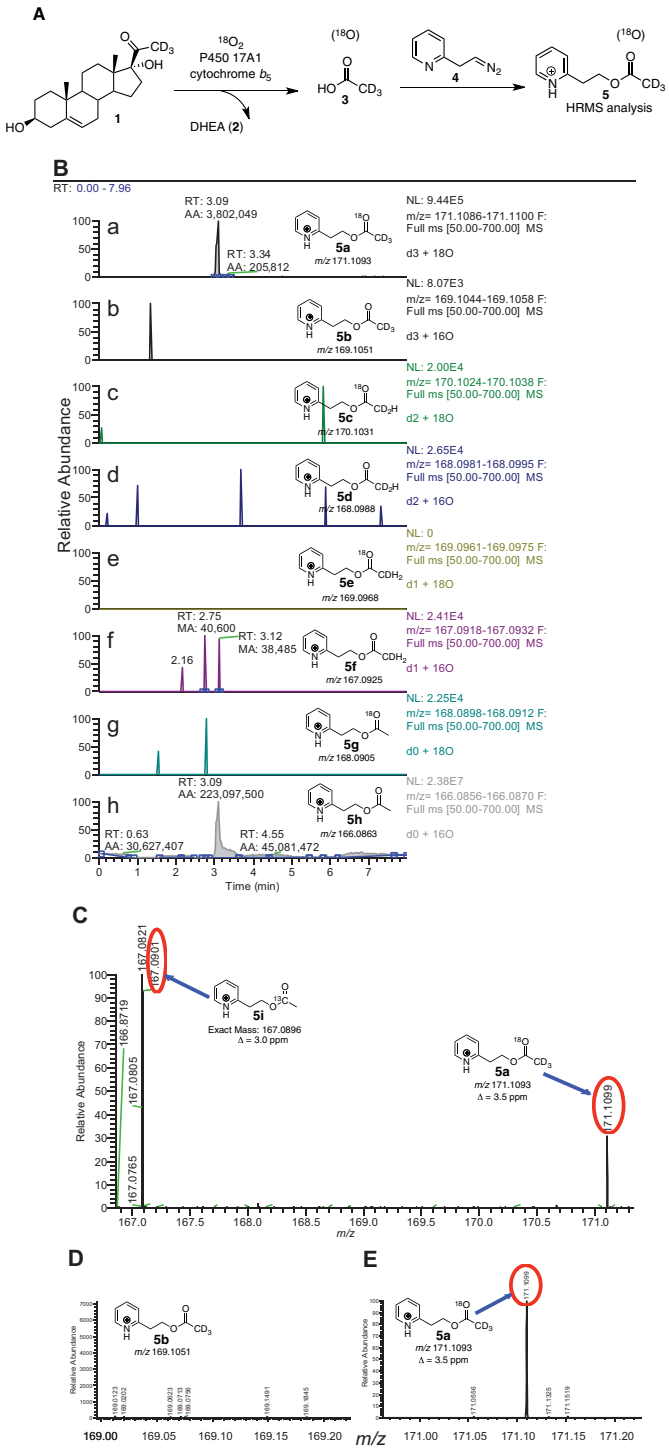


FIGURE 4. **P450 17A1 incubation with [21,21,21-$^2H_3$]17α-hydroxypregnenolone (*1*) in the presence of $^{18}O_2$ followed by derivatization and analysis by HRMS.** *A*, scheme showing the incubation of deuterated lyase substrate (***1***) with P450 17A1 and cytochrome b_5 in the presence of $^{18}O_2$. The acetic acid product (***3***) was derivatized with the diazoethylpyridine reagent (***4***) and analyzed by liquid chromatography-HRMS. *B*, ion chromatograms monitoring the various isotopically labeled acetate products that were derivatized to the pyridylethyl esters (***5a–h***), with 4 ppm mass tolerance parameter. *a*, *m/z* 171 window (d_3, ^{18}O); *b*, *m/z* 169 window (d_3, ^{16}O); *c*, *m/z* 170 window (d_2, ^{18}O); *d*, *m/z* 168 window (d_2, ^{16}O); *e*, *m/z* 169 window (d_1, ^{18}O); *f*, *m/z* 167 window (d_1, ^{16}O); *g*, *m/z* 168 window (d_0, ^{18}O); *h*, *m/z* 166 window (d_0, ^{16}O). *C*, mass spectrum of the *m/z* 166.5–171.3 range by selecting the t_R 3.01–3.12-min time interval in the ion chromatogram corresponding to the pyridine ester retention time. Shown at *m/z* 167.0901 is the peak corresponding to the acetate from background acetic acid from the natural abundance of ^{13}C isotope (***5i***, expected mass, *m/z* 167.0896, Δ 3.0 ppm). The peak at *m/z* 171.1099 corresponds to the acetate derived from the enzymatic product (***5a***, expected mass, *m/z* 171.1093, Δ 3.5 ppm). *D*, expansion of the mass spectrum (*m/z* 168.95–169.22) from *C* showing the absence of d_3-labeled acetate with no ^{18}O incorporation (***5b***, expected mass, *m/z* 169.1051). *E*, expansion of the mass spectrum (*m/z* 170.95–171.22) from *C* showing the presence of d_3-labeled acetate with ^{18}O incorporation (***5a***, expected mass, *m/z* 171.1093). *p*, profile (peaks are shown in profile mode and not "centroid"). *ESI*, electrospray ionization; *RT*, retention time; *NL*, normalized level. More information about the meaning of the settings can be obtained from the Xcalibur Qual Browser User Guide (Thermo Scientific).

P450 17A1 Reactions with Iodosylbenzene—Preliminary experiments indicated that the most effective concentration to use was 300 μM (results not presented).

Two products were formed from 17α-hydroxyprogesterone in both the iodosylbenzene and NADPH-based systems (Fig. 6). The expected product androstenedione (Fig. 1) was characterized by co-elution with a standard and by both LC-UV and LC-MS comparisons with a standard (data not shown). The other product, which eluted just before androstenedione, was identified as 16,17α-dihydroxyprogesterone by co-elution with a standard and by both LC-UV and LC-MS comparisons with a reference standard (Fig. 7). Although the dihydroxy product co-eluted with the 16α,17α-diastereomer, we cannot exclude the presence of the 16β-stereoisomer.

The products formed from 17α-hydroxypregnenolone were converted to Δ^4 steroids by the action of cholesterol oxidase. These were identified as 16,17α-dihydroxyprogesterone and androstenedione, thus indicating that the products formed from 17α-hydroxypregnenolone were 16,17α-dihydroxypregnenolone and DHEA.

The rates of formation of 16,17α-dihydroxyprogesterone and androstenedione from 17α-hydroxyprogesterone in the NADPH- and iodosylbenzene-based systems were comparable in the absence of cytochrome b_5 (Fig. 8*A*). The iodosylbenzene-dependent reaction was stimulated 2-fold by cytochrome b_5, but the stimulation of the reaction that used NADPH-P450 reductase was much greater (10-fold), so that the iodosylbenzene *versus* NADPH-P450 reductase comparison (with cytochrome b_5 present) is more disparate (Fig. 8*A*).

With 17α-hydroxypregnenolone as substrate, a similar conclusion was reached regarding comparisons of the rates of the NADPH/reductase- and iodosylbenzene-supported reactions (Fig. 8*B*). When the 16-hydroxylation of the 17α-hydroxy steroids was considered, the iodosylbenzene-supported reactions were faster (Fig. 9). It is also notable that these reactions were stimulated by cytochrome b_5.

Reactions with H_2O_2—H_2O_2 was added to purified P450 17A1 (with cytochrome b_5 present), and no detectable 17α,20-lyase activity was found toward 17α-hydroxypregnenolone or 17α-hydroxyprogesterone, using varying concentrations of H_2O_2 (up to 10 mM) (Fig. 9). Under these conditions, the usual reconstituted P450 17A1/NADPH-P450 reductase/cytochrome b_5 system yielded the expected products (Fig. 10, *B* and *E*).

Additional Oxidation Products—With both 17α-hydroxyprogesterone and 17α-hydroxypregnenolone, the rates of formation of the lyase products (androstenedione and DHEA) were no longer linear after 5 min (300 s) (Fig. 8). The change was

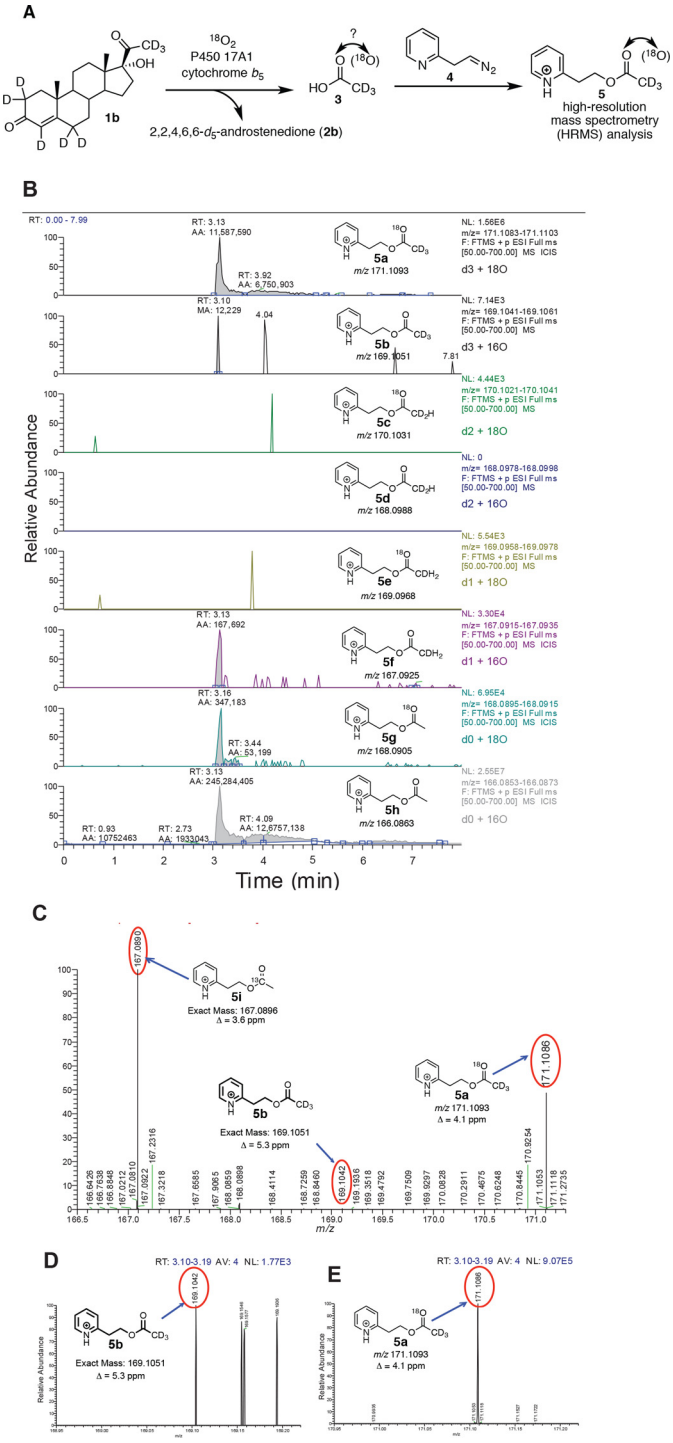


FIGURE 5. **P450 17A1 incubation with 17α-hydroxy-[2,2,4,6,6,21,21,21-$^2H_8$]progesterone (*1b*) in the presence of $^{18}O_2$ followed by derivatization and analysis by HRMS.** *A*, scheme showing the incubation of deuterated lyase substrate (***1b***) with P450 17A1 and cytochrome b_5 in the presence of $^{18}O_2$. The acetic acid product (***3***) was derivatized with the diazoethylpyridine reagent (***4***) and analyzed by liquid chromatography-HRMS. *B*, ion chromatograms monitoring the various isotopically labeled acetate products that were derivatized to the pyridylethyl esters (***5a–h***), with 6 ppm mass tolerance parameter. *a*, *m/z* 171 window (d_3, ^{18}O); *b*, *m/z* 169 window (d_3, ^{16}O); *c*, *m/z* 170 window (d_2, ^{18}O); *d*, *m/z* 168 window (d_2, ^{16}O); *e*, *m/z* 169 window (d_1, ^{18}O); *f*, *m/z* 167 window (d_1, ^{16}O); *g*, *m/z* 168 window (d_0, ^{18}O); *h*, *m/z* 166 window (d_0, ^{16}O). *C*, mass spectrum of the *m/z* 166.5–171.3 range by selecting the t_R 3.10–3.19-min time interval in the ion chromatogram corresponding to the pyridine ester retention time. Shown at *m/z* 167.0890 is the peak corresponding to the acetate from background acetic acid from the natural abundance of ^{13}C isotope (***5i***, expected mass, *m/z* 167.0896, Δ 3.6 ppm). The peak at *m/z* 171.1099 corresponds to the acetate derived from the enzymatic product (***5a***, expected mass, *m/z* 171.1093, Δ 4.1 ppm). *D*, expansion of the mass spectrum (*m/z* 168.95–169.22) (from *C*) showing the detection of d_3-labeled acetate with no ^{18}O incorporation (***5b***, expected mass, *m/z* 169.1051, Δ 5.3 ppm). *E*, expansion of the mass spectrum (*m/z* 170.95–171.22) from *C* showing the presence of d_3-labeled acetate with ^{18}O incorporation (***5a***, expected mass, *m/z* 171.1093). *p*, profile (peaks are shown in profile mode and not "centroid"). *ESI*, electrospray ionization. *RT*, retention time. *NL*, normalized level. More information about the meaning of the settings can be obtained from the Xcalibur Qual Browser User Guide (Thermo Scientific).

more obvious in the latter case, with the amount of accumulated product decreasing (Fig. 8*B*). The phenomenon was found to be the result of further 16-hydroxylation of the lyase products, in that we were able to identify these products (t_R, UV spectra, and mass spectra) in the longer term reactions with both substrates (with 16-hydroxy-DHEA being converted to 16-hydroxyandrostenedione by cholesterol oxidase in the assays with 17α-hydroxypregnenolone) (Fig. 11). The time course of formation of these products from androstenedione and DHEA is shown in Fig. 12.

We also noted a decrease in the level of 16,17α-dihydroxy steroids with extended time (in the NADPH-supported reactions, Fig. 9). We analyzed the products formed from (commercial) 16α,17α-dihydroxyprogesterone. One product was 16α-hydroxyandrostenedione, identified above (Fig. 13). The major product formed from 16α,17α-dihydroxypregnenolone was 16α-hydroxy-DHEA, identified by its mass and NMR spectra (Fig. 14).

Identification of Steroid B Ring Hydroxylation Product—The other major product in the incubation of 16α,17α-dihydroxyprogesterone with P450 17A1 was a triol, as judged by HRMS (*m/z* 363.2160, calculated MH^+ *m/z* 363.2166, Δ 1.7 ppm) (Fig. 15*A*) (42). The UV spectrum was similar to those of other Δ^4 3-keto steroids except blue-shifted ~5 nm (Fig. 15*B*), consistent with an intact Δ^4 3-keto steroid having some modification near the chromophore. The site of hydroxylation was identified as C-6 by ^1H NMR (Fig. 15*C* and Table 1), *i.e.* 6β,16α,17α-trihydroxyprogesterone (43, 44). The 18, 19, and 21 methyl groups were intact (δ 0.79, 1.40, and 2.28 ppm), but there was a new multiplet at δ 4.38 ppm. The NOESY spectrum (supplemental Fig. S1) showed a spatial correlation between the new hydroxymethine proton (δ 4.38 ppm) and the Δ^4-proton (δ 5.70 ppm). Moreover, the HMBC spectrum (heteronuclear multiple-bond correlation (NMR) spectroscopy) (supplemental Fig. S2) indicated a 3-bond coupling interaction between the C4-carbon (δ 125 ppm) and the hydroxymethine proton (δ 4.38 ppm) suggesting either the C2-position or the C6-position for the newly identified hydroxymethine proton (δ 4.38 ppm). The C6-position for the hydroxymethine proton (δ 4.38 ppm) was established because the C2-methylene protons (δ 2.53 and 2.42 ppm), which had a 2-bond correlation to the C3-keto carbon atom (δ 205.1 ppm) in the HMBC spectrum, were present. Moreover, the COSY spectrum (supplemental Fig. S3) indicated a 3-bond coupling between the C6-proton (δ 4.38 ppm) and the C7-protons (δ 1.98 and 1.34 ppm) (see also HSQC spectrum, supplemental Fig. S4).

We have assigned the stereochemistry of the C6-hydroxyl group as β, based on the chemical shifts of the 7-protons of the

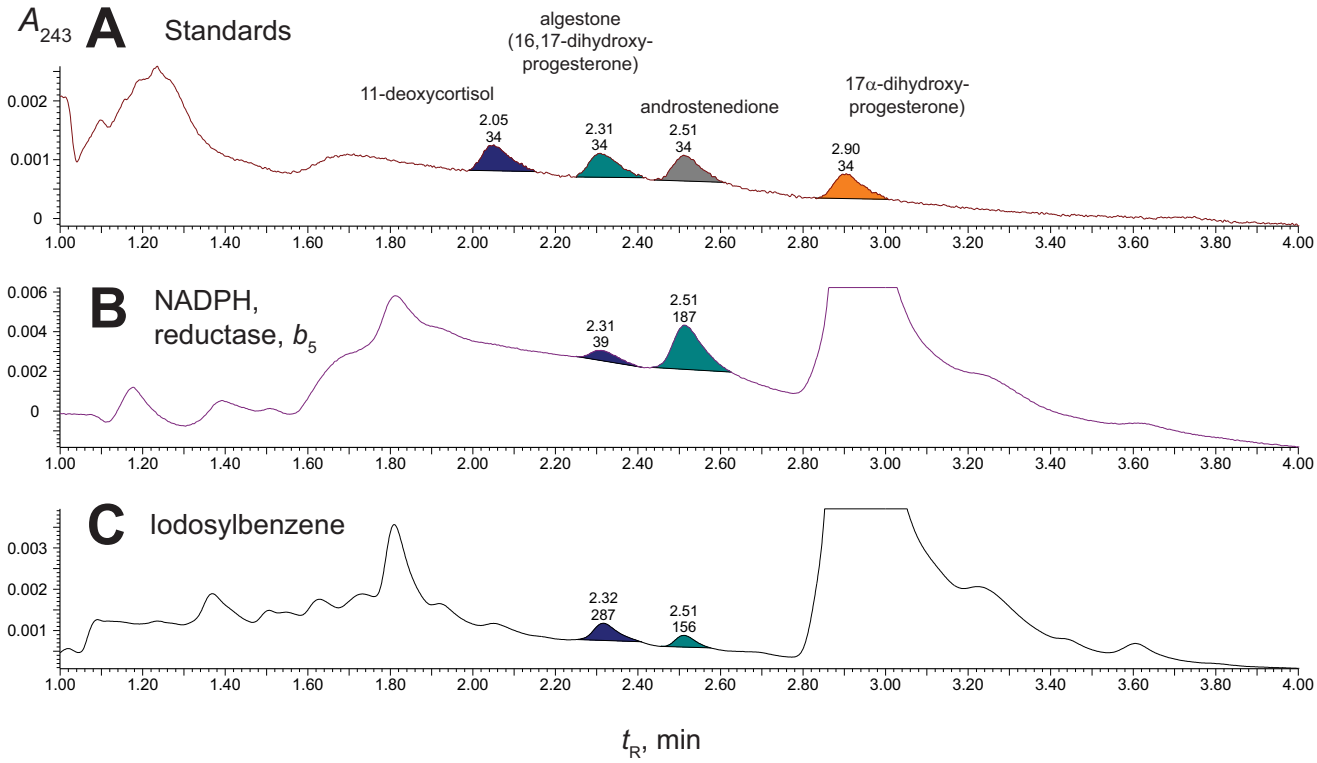


FIGURE 6. **Formation of 16,17α-dihydroxyprogesterone and androstenedione from 17α-hydroxyprogesterone by P450 supported by the oxygen surrogate iodosylbenzene.** Retention times (t_R) and integration units are indicated on the chromatograms. *A*, standard compounds. *B*, reaction (0.5 μM P450 17A1) supported by NADPH-P450 reductase (2.0 μM), cytochrome b_5 (0.5 μM), and NADPH (30-s incubation). *C*, reaction (0.5 μM P450 17A1 and cytochrome b_5 (0.5 μM)) with 0.30 mM iodosylbenzene (30-s incubation). In control experiments with only cytochrome b_5 and iodosylbenzene (2 mM) mixed with the 17α-hydroxysteroids, the amounts of androstenedione detected were <15% of the amounts observed in this and similar studies with both 17α-hydroxysteroids.

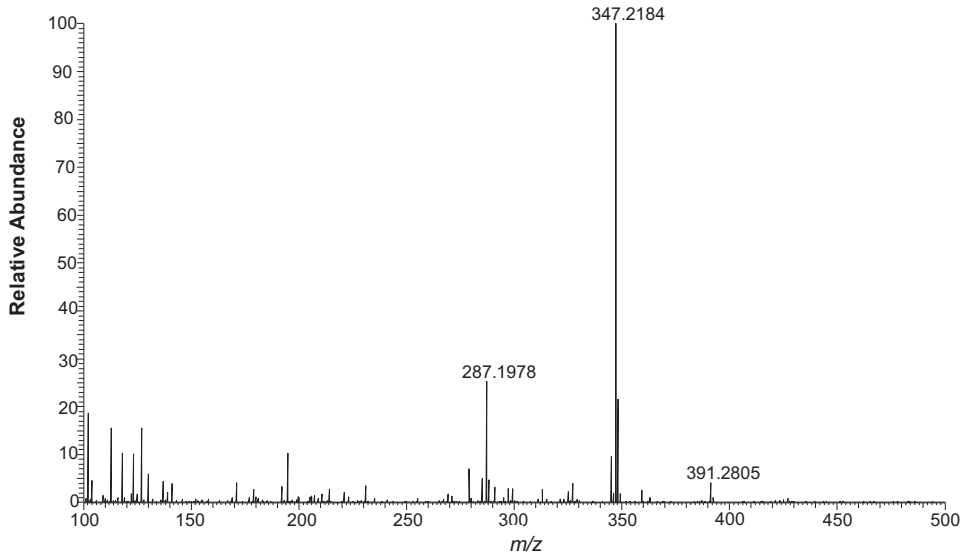


FIGURE 7. **Identification of 16,17α-dihydroxyprogesterone as a product of 17α-hydroxyprogesterone.** HRMS spectrum of 16,17-dihydroxyprogesterone formed in a reaction with NADPH-P450 reductase, cytochrome b_5, and NADPH. Exact mass 346.2217 (protonated species): observed for MH^+, *m/z* 347.2184 (Δ 9.5 ppm).

steroid. In considering the chemical shifts of literature compounds (see Refs. 61 and 62 and see Table 2C in Ref. 42 and references therein), corresponding to 6α- and 6β-hydroxyprogesterone), the chemical shifts of the 7α- and 7β-protons are very informative. In the isolated P450 17A1 product, the chemical shifts of the 7α- and 7β-protons were (δ) 1.34 and 1.98 ppm, respectively, and in Ref. 42, the 7α- and 7β-protons were at (δ) 1.28 and 2.02 for 6β-hydroxyprogesterone, whereas the protons had chemical shifts of (δ) 1.11 and 2.19 for 6α-hydroxyprogesterone. Thus, 6β-hydroxy is the most likely stereochemistry of the new product. The NOESY spectrum showed spatial correlation between the C6-proton (δ 4.38 ppm) and both of the

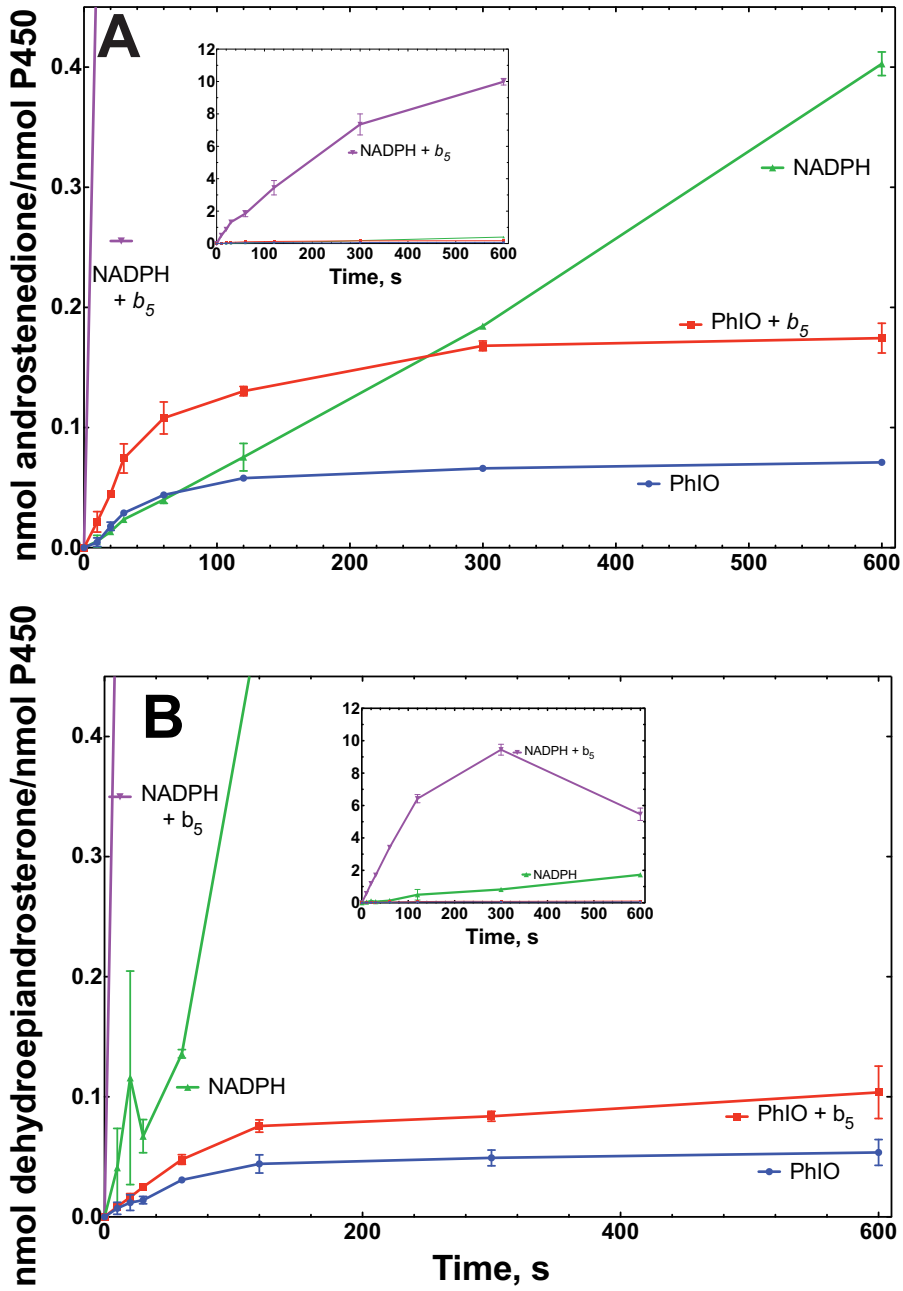


FIGURE 8. **Time course and effect of cytochrome b_5 on 19-carbon steroid formation in the presence of iodosylbenzene (*PhIO*) or the typical NADPH-supported reaction.** *A*, oxidation of 17α-hydroxyprogesterone. *B*, oxidation of 17α-hydroxypregnenolone. The *insets* show the NADPH-supported reactions in the presence of cytochrome b_5. The *points* are means of duplicate assays, shown as means ± range.

7α- and 7β-protons (δ 1.34 and 1.98 ppm). Considering a Newman projection down the C6–C7 bond axis, this NOESY interaction is supported by the 6β-hydroxy configuration (supplemental Fig. S1).

We also analyzed the products formed from 16α,17α-dihydroxypregnenolone. One product was 16α-hydroxy-DHEA. The other product was either a tetraol or an epoxytriol (5,6-epoxy-3β,16α,17α-trihydroxypregnan-20-one), as judged by HRMS (*m/z* 365.2305, calculated mass, *m/z* 365.2323, Δ 4.9 ppm). The site of oxygen incorporation was not identified. The major product isolated from this reaction was 16α-hydroxy-DHEA, as can be seen from the ^{1}H NMR spectrum of the purified product (Fig. 14*B*). There is a loss of the C21-methyl protons (δ 2.25 ppm) and an upfield shift of the 16β-proton (5.1 ppm to 4.4 ppm) (Fig. 14*B*). The proton NMR spectrum of the isolated P450 17A1 product also matched a previously reported NMR spectrum of synthetic 16α-hydroxy-DHEA (45).

Solvent Kinetic Isotope Effects on 17α,20-Lyase Reactions—No C–H bond-breaking steps are involved in any steps proposed in Fig. 3 except for Fig. 3*D*, which is not supported by the ^{18}O work. Thus, no C-H kinetic deuterium isotope effect studies can be applied, but solvent kinetic isotope effect experiments can be informative.

No solvent kinetic isotope effect was found for the 17α,20-lyase reaction with 17α-hydroxyprogesterone (measured rates of 1.47 ± 0.03 min^{-1} in H_2O and 1.40 ± 0.03 min^{-1} in 95% D_2O

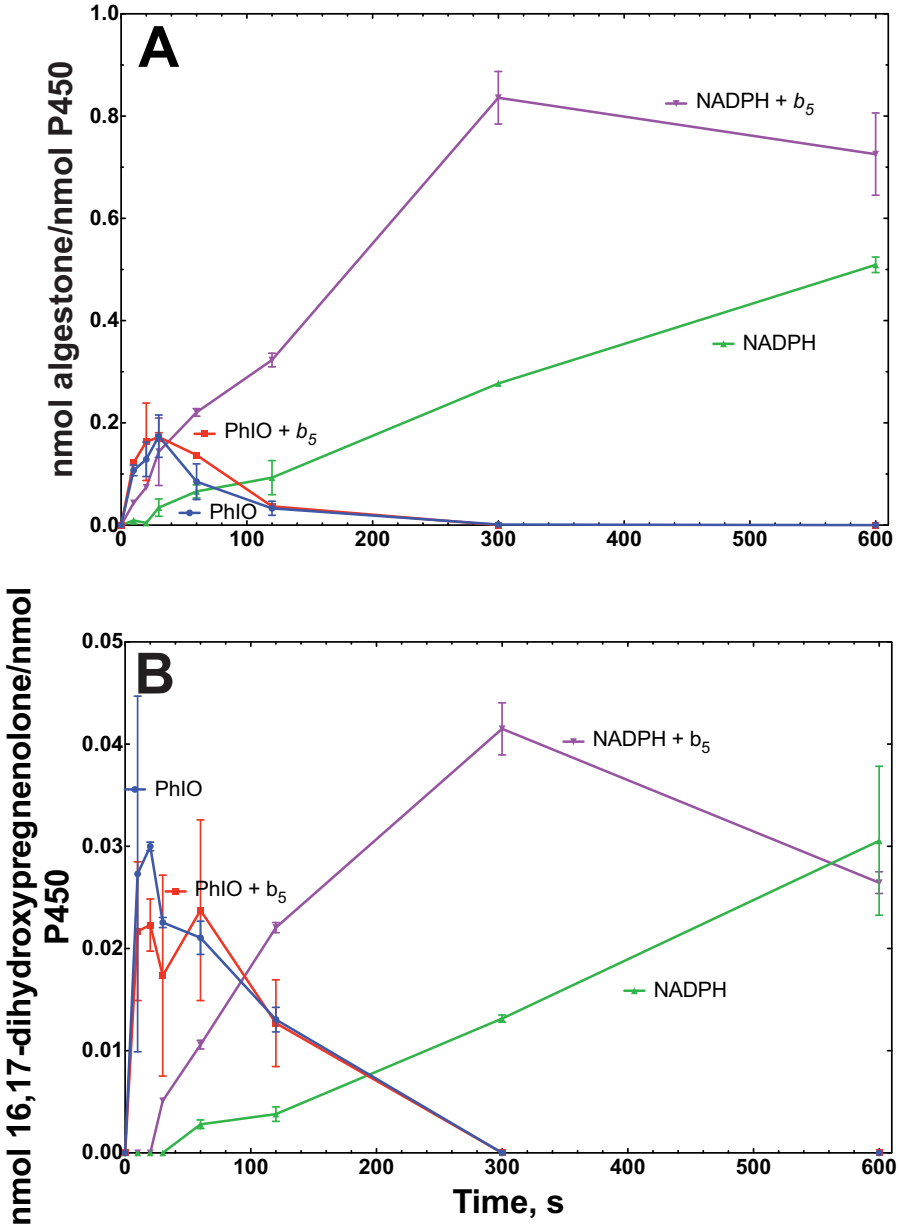


FIGURE 9. **Time course and effect of cytochrome b_5 on steroid 16-hydroxylation in the presence of iodosylbenzene (*PhIO*) or the typical NADPH-supported reaction.** *A*, oxidation of 17α-hydroxyprogesterone. *B*, oxidation of 17α-hydroxypregnenolone. The *points* are means of duplicate assays, shown as means ± range.

(v/v), $n = 4$, calculated isotope effect of 1.05 ± 0.09 (S.D.)). In contrast, we observed a small but repeatable inverse isotope effect (0.83 ± 0.05 (S.D.), $n = 4$) for the 17α,20-lyase reaction with 17α-hydroxypregnenolone under the usual conditions with cytochrome b_5, NADPH-P450 reductase, and substrate concentration of 30 μM (measured rates of 4.10 ± 0.22 min^{-1} in H_2O, 4.94 ± 0.13 min^{-1} in 95% D_2O (v/v), calculated from four independent experiments (± S.D.)) (Figs. 16 and 17). The solvent kinetic deuterium isotope effects for the 16-hydroxylation reactions were 1.35 ± 0.15 for 17α-hydroxyprogesterone and 1.34 ± 0.23 for 17α-hydroxypregnenolone ($n = 4$, ± S.D., data not shown).

Discussion

Our results indicate that acetic acid recovered in the human P450 17A1 reactions with either 17α-hydroxyprogesterone or 17α-hydroxypregnenolone contained ^{18}O atom from molecular oxygen. These results are consonant with the original analysis of Akhtar and co-workers on P450 17A1 (27, 28) and, on their own, are consistent with the FeO_2^- mechanism presented in Fig. 3*A* (40, 46). Alternative mechanisms that are still consistent with the ^{18}O labeling results, but involving FeO^{3+}, are presented in Fig. 3, *B*, *C*, and *F*, *arrow b* (7, 40, 47). The mechanisms in Fig. 3, *B* and *C* involve formation of an acetyl radical, which has adequate chemical precedent (32, 48–50). The dioxetane mechanism is similar to one that has been proposed for tryptophan and indole dioxygenases (51). One of these two FeO^{3+} mechanisms is proposed to contribute to the lyase reaction in that (i) iodosylbenzene can support the lyase reaction, and (ii) we report that P450 17A1-17α-hydroxysteroid complexes are poised for multiple hydroxylation reactions in addition to lyase reactions.

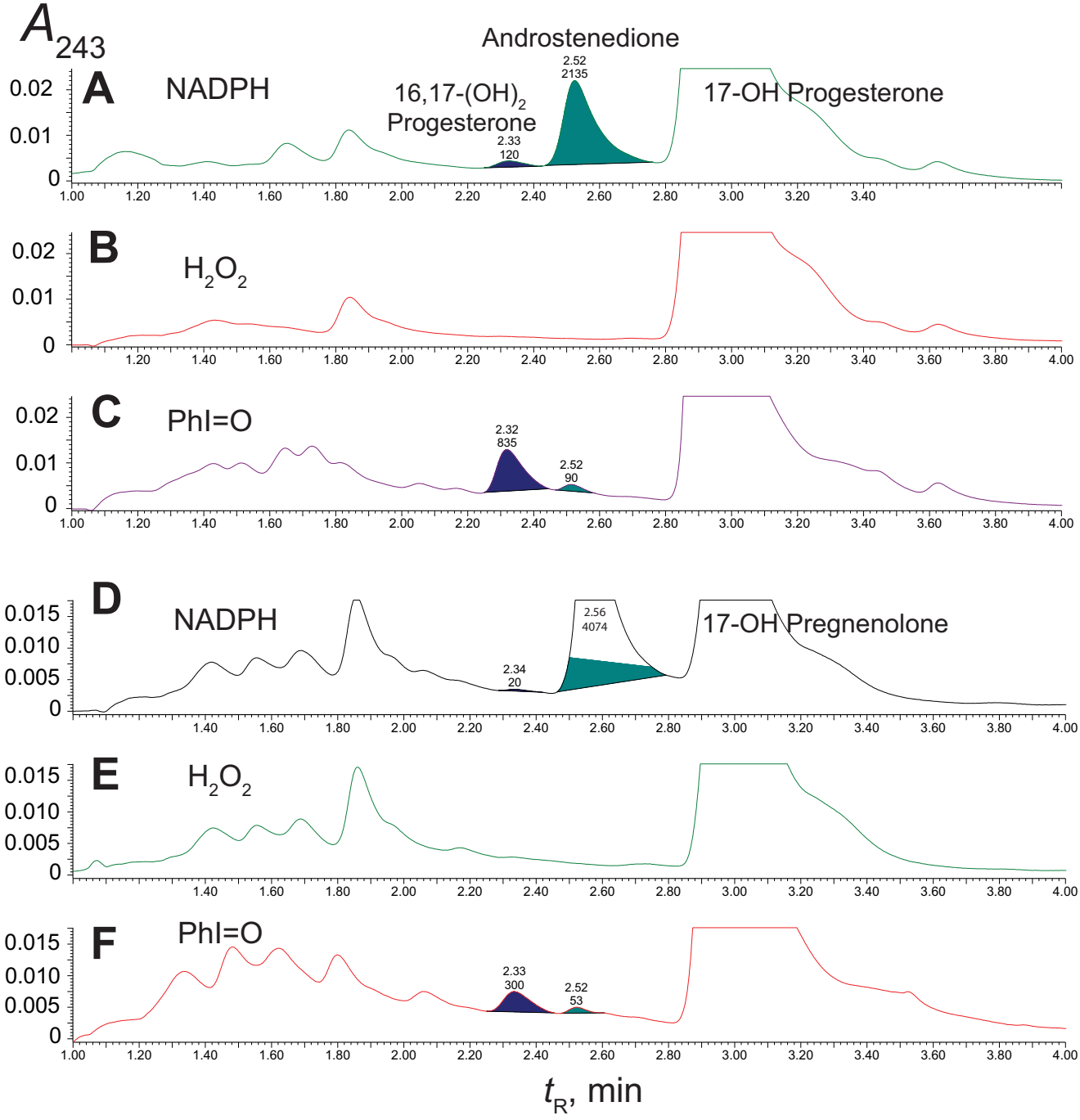


FIGURE 10. **Reaction products formed from 17α-hydroxyprogesterone and 17α-hydroxypregnenolone in P450 17A1 reactions supported by various factors.** Retention times (t_R) and integration units are indicated on the chromatograms. *A–C*, 17α-hydroxyprogesterone; *D–F*, 17α-hydroxypregnenolone. *A* and *D*, NADPH-P450 reductase, cytochrome b_5, and NADPH; *B* and *E*, H_2O_2 (10 mM) (with cytochrome b_5); *C* and *F*, iodosylbenzene (*PhI=O*, 300 μM) (with cytochrome b_5). In these studies the Δ^5 products (formed from 17α-hydroxypregnenolone) were oxidized to Δ^4 products to facilitate LC-UV analysis.

Incubations with $^{18}O_2$—Previous studies of $^{18}O_2$ incubations with P450 17A1 concluded that ^{18}O incorporation into the acetic acid product was the *major* isotopologue detected, leading to the conclusion that the ferric peroxide was the iron-active species for C–C bond cleavage (26, 52). However, these studies (i) used low resolution mass spectrometry and (ii) used microsomes from porcine testes as the source of enzyme (*i.e.* non-purified enzyme); and (iii) the report that used the direct lyase substrate 17α-hydroxy-[21,21,21-$^2H_3$]pregnenolone did not present raw data (26), whereas a subsequent report used [16α,17α,21,21,21-$^2H_5$]pregnenolone as the substrate and not the direct substrate that results in the formation of DHEA, *i.e.* 17α-hydroxypregnenolone (52). Furthermore, the low resolution mass spectrometry used in these studies yielded an ambiguous determination of ^{18}O-, ^2H-, and ^{13}C-labeled content of the acetate products (52), which resulted in the interpretation of multiple possible mechanisms for the lyase step of P450 17A1 (Fig. 3). We performed the incubation with purified P450 17A1

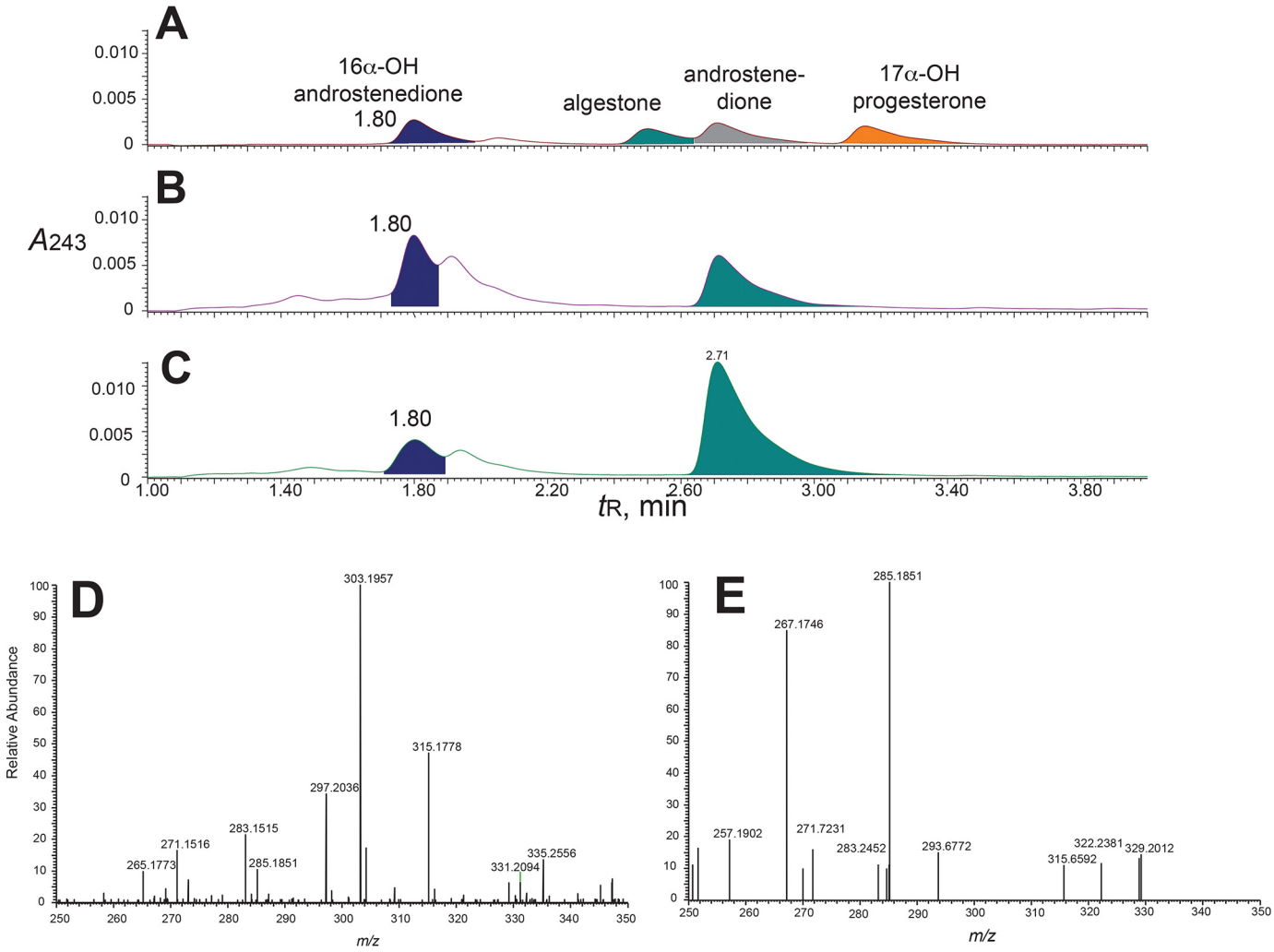


FIGURE 11. **Identification of 16-hydroxy steroids as reaction products formed from DHEA and androstenedione.** *A*, authentic steroid standards: 16α-hydroxyandrostenedione, algestone (16α,17α-dihydroxyprogesterone), androstenedione, and 17α-hydroxyprogesterone; *B*, 10-min DHEA incubation (with products treated with cholesterol oxidase); *C*, 10-min androstenedione incubation; *D*, mass spectrum of peak identified as 16-hydroxyandrostenedione (formed from androstenedione); *E*, MS/MS analysis of *m/z* 303.2 peak of *D*.

and labeled lyase substrates (17α-hydroxy-[21,21,21-$^{2}H_3$] pregnenolone and 17α-hydroxy-[2,2,4,6,6,21,21,21-$^{2}H_8$]progesterone) in the presence of $^{18}O_2$ and analyzed the acetate product by HRMS after derivatization with the new diazo reagent. Our results unambiguously established that the acetate produced from the enzymatic incubation incorporated one oxygen atom from molecular oxygen in both cases, with the C-21 deuteriums retained (Figs. 4 and 5). Moreover, we did not detect significant amounts of any other acetate isotopologues from the enzyme incubation (*i.e.* loss of one deuterium or lack of ^{18}O incorporation). These experiments agree with the observation of oxygen incorporation from the reports of Akhtar and co-workers (26, 52). However, although these data can support a ferric peroxide mechanism for C–C bond cleavage (Fig. 3*A*), they do not rule out a compound I mechanism (Fig. 3, *B, C,* and *F, arrow b*).

Oxygen Surrogate Studies, Iodosylbenzene—The use of iodosylbenzene and NADPH-P450 reductase to form compound I with P450 17A1 resulted in two different activities when the 17α-hydroxysteroid was used as the substrate. In both conditions, 16α-hydroxylation and C–C bond cleavage activities toward 17α-hydroxyprogesterone yielded 16,17α-dihydroxyprogesterone and androstenedione, respectively (Figs. 6, 8*A*, and 9*A*). However, the product distributions were different; iodosylbenzene yielded more 16-hydroxylation relative to C–C bond cleavage (~9:1, Fig. 6*C*) compared when NADPH-P450 reductase was used (~0.1:1, Fig. 6*B*). The switch in reactivities depending on the oxidation system used (iodosylbenzene *versus* NADPH-P450 reductase) suggests a conformational change in the enzyme-substrate complex when the reductase binds to the P450 enzyme. Moreover, when 17α-hydroxypregnenolone was used as the substrate, the 16-hydroxylation activity was diminished (Fig. 10, *D* and *F*) relative to when 17α-hydroxyprogesterone was used as the substrate. Similarly, this substrate-dependent switch in reactivity is reminiscent of the different 16- *versus* 17-hydroxylation regioselectivities observed when two different substrates, progesterone and pregnenolone, are used for P450 17A1 (53), which is explained by the 3-keto-Δ^4 *versus* 3β-hydroxy-Δ^5 moieties in the AB-ring systems of these steroid substrates.

Moreover, the fact that P450 17A1 is catalyzing a C-H hydroxylation with its lyase substrate, 17α-hydroxypregnenolone,

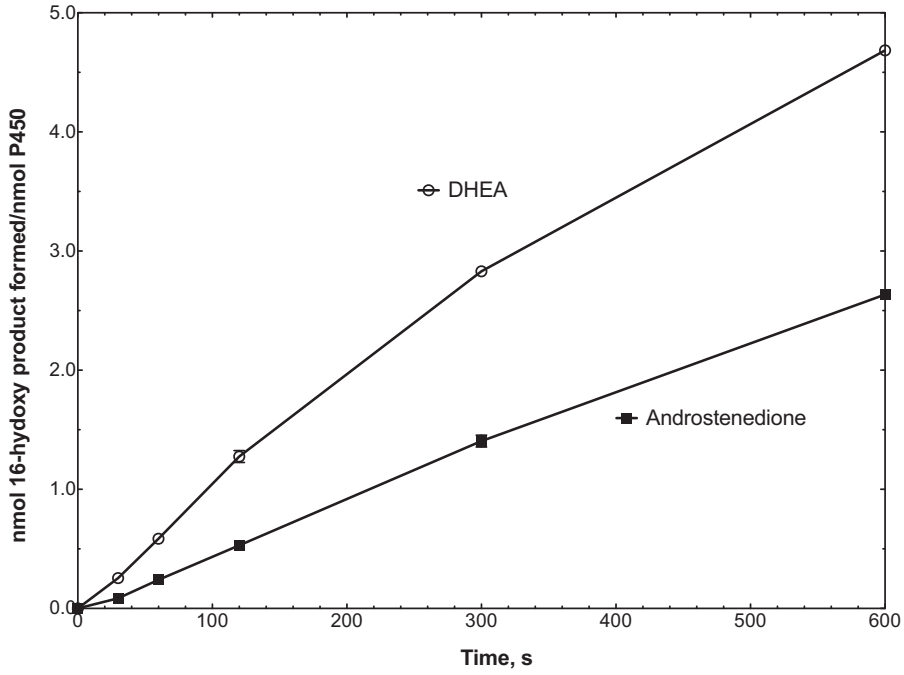


FIGURE 12. **Time course of 16-hydroxylation of androstenedione and DHEA by P450 17A1.**

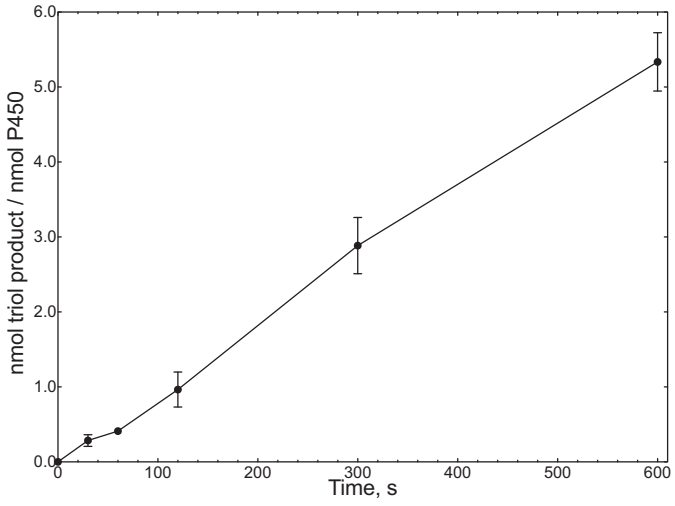


FIGURE 13. **Rate of conversion of 16α,17α-dihydroxyprogesterone to 6β,16α,17α-trihydroxyprogesterone (Fig. 15) by P450 17A1.** The *points* are means of duplicate assays, shown as means ± range.

supports the presence of a compound I species, which either hydroxylates the C16-position or cleaves the C17,C20-bond as shown in Fig. 3. This observation may contradict the conclusions about the active iron hydroperoxo species observed by resonance Raman spectroscopy (9, 25). However, it is possible that the iron peroxohemiketal species reported in the resonance Raman study (9), *i.e.* Fig. 3*A*, tetrahedral intermediate, was a structural misassignment and that the actual observed species was indeed an iron peroxo intermediate attached through the C17-position of the steroid (Fig. 3*F*). This iron peroxo intermediate can be formed from a nucleophilic attack of the C17-hydroxy group of the lyase substrate (*i.e.* 17α-hydroxypregnenolone or 17α-hydroxyprogesterone, Fig. 3*F*) onto compound I.

It should be pointed out that the iodosylbenzene mechanism may be more complex than just a direct oxygen transfer, as pointed out by Ortiz de Montellano (46). A possible intermediate is shown in Fig. 18, which may even have oxidant capacity of its own.

Oxygen Surrogate Studies, Hydrogen Peroxide—Many studies in the literature involve the use of alkyl hydroperoxides as oxygen surrogates for P450 reactions, beginning with Kadlubar *et al.* (54). However, although peracids can be used as reagents to generate P450 compound I (55), studies with alkyl hydroperoxides are problematic due to the production of radicals and their ensuing chemistry (46, 56). Some bacterial family 152 P450s appear to use H_2O_2 as a physiological cofactor (37, 57–59), and bacterial P450 101A1 ($P450_{cam}$) was mutated to a species that could efficiently utilize H_2O_2 in reactions (60). H_2O_2 can support some mammalian P450 reactions after direct addition (61–66) (although generally not as well as alkyl hydroperoxides (54)), but the role of a ferric peroxide in each oxygenation reaction can only be postulated, in that the ferric peroxide can subsequently convert to compound I.

In principle, the FeO_2^- complex could proceed to compound I (FeO^{3+}) through appropriate acid-base catalysis, but there are also side reactions that may diminish the progress of a putative Fe^{3+}-H_2O_2 complex on to FeO^{3+} (Fig. 2). We made attempts to observe compound I or other intermediates by mixing P450 17A1 with H_2O_2 (10 mM) or iodosylbenzene (300 μM) in a stopped-flow spectrophotometer (dead time ~2 ms, rapid scanning) but were unsuccessful in seeing any distinct complexes (data not presented). However, given the difficulties encountered by others in observing these transient species even with bacterial P450s (55), negative results are inconclusive.

Cytochrome b_5 Effects—Another issue that is still not resolved is the stimulatory effect of cytochrome b_5, which is known to

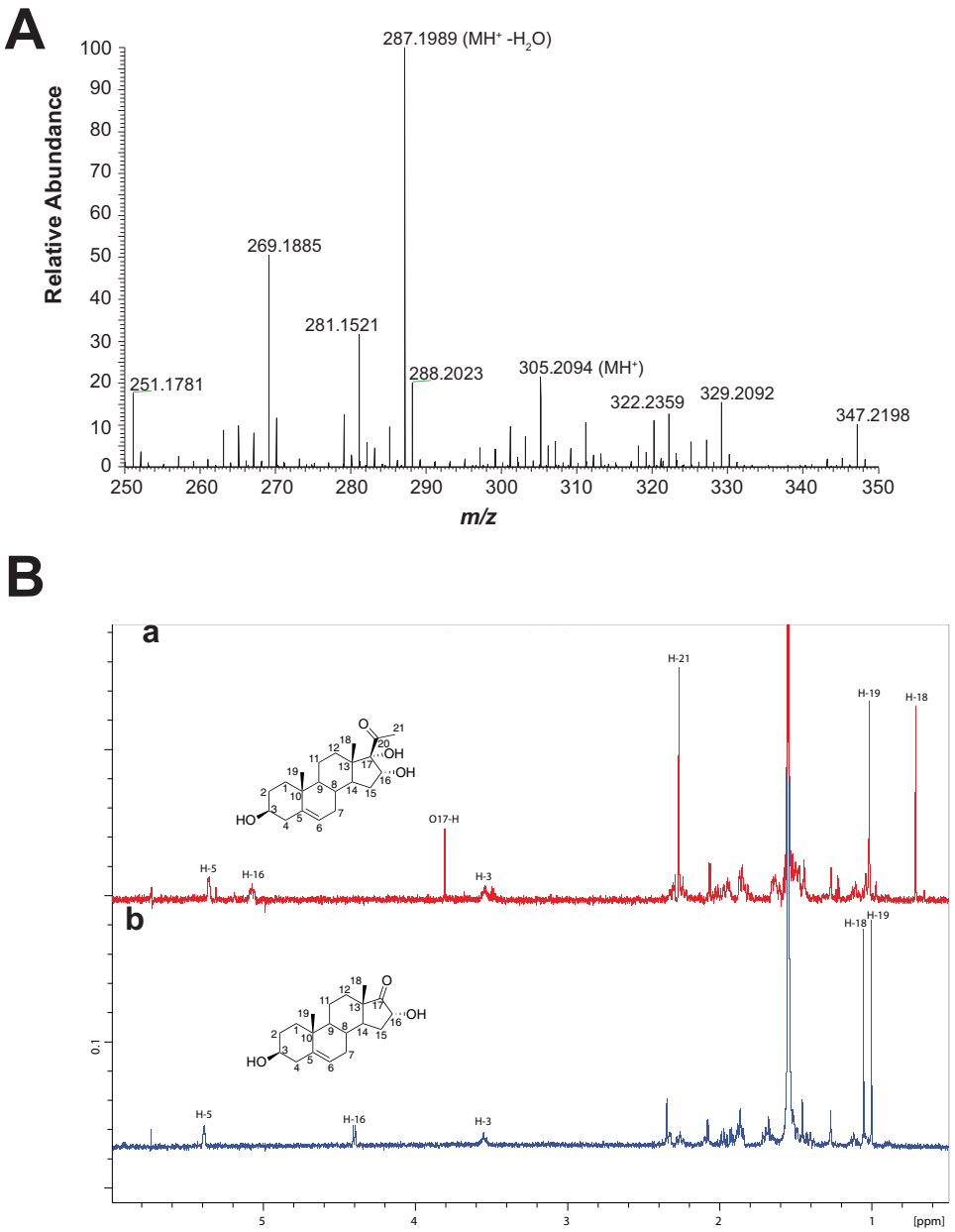


FIGURE 14. **Characterization of 16α-hydroxy-DHEA.** The product was formed in an incubation of an NADPH-reconstituted P450 17A1 system with 16α,17α-dihydroxypregnenolone and isolated by preparative HPLC. *A*, HRMS spectrum of $[DHEA + 16]^+$ peak, 16α-hydroxy-DHEA (theoretical *m/z* for MH^+ 305.2111, found *m/z* 305.2094). *B*, NMR spectra of 16α,17α-dihydroxypregnenolone (*a*) and product (*b*) in $CDCl_3$ (600 MHz). See text for discussion.

promote the 17α,20-lyase reaction of P450 17A1. Results with apo-cytochrome b_5, devoid of heme, have shown that cytochrome b_5 does not donate the second electron in the catalytic cycle of this P450 (67). The stimulation of 17α,20-lyase activity by cytochrome b_5 in the iodosylbenzene-supported reaction (Fig. 8) is consistent with this view. We also note that cytochrome b_5 stimulated the 16-hydroxylation reactions with both 17α-hydroxyprogesterone and 17α-hydroxypregnenolone (Fig. 8). In other unpublished data,[4] we have also noted the stimulation of the 17α-hydroxylation of both progesterone and pregnenolone by cytochrome b_5. Our conclusion about the stimulatory role of cytochrome b_5 in the iodosylbenzene reactions is that it is acting in an allosteric manner to facilitate these reactions (*e.g.* due to more ideal juxtaposition in reaction intermediates), which is the reason proposed for the normal physiological reaction (6, 7, 67).

Hydroxylations Catalyzed by P450 17A1—The 16α-hydroxylation of DHEA has previously been reported to be catalyzed by P450 3A4 (68, 69). Upon monitoring the production of DHEA from 17α-hydroxypregnenolone by P450 17A1 over time (Fig. 8*B*, *inset*), there was a decrease in DHEA formation in the time points greater than 5 min. This observation suggested that DHEA was further being oxidized to another product. We hypothesized that this new product would correspond to 16-hydroxy-DHEA based on the other activities of P450 17A1 (16-hydroxylation of progesterone (53) and 16-hydroxylation

[4] E. Gonzalez and F. P. Guengerich, unpublished data.

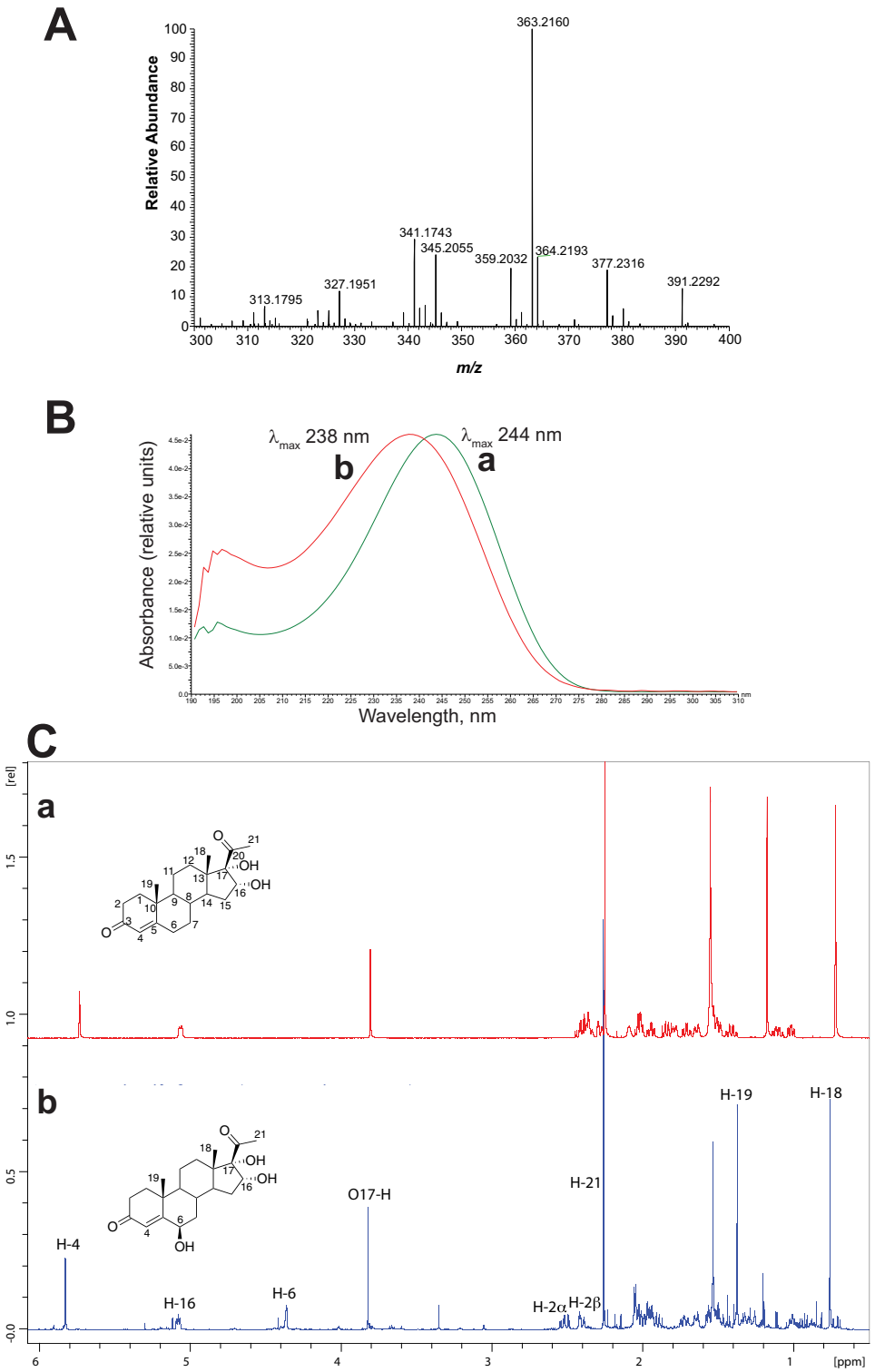
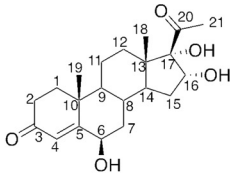


FIGURE 15. **Characterization of 6β,16α,17α-trihydroxyprogesterone.** The product was formed in an incubation of an NADPH-reconstituted P450 17A1 system with 16α,17α-dihydroxyprogesterone and isolated by preparative HPLC. *A*, HRMS spectrum (theoretical *m/z* for MH^+ 363.2166, found *m/z* 363.2160). *B*, UV spectra of product (*b*) compared with 16α,17α-dihydroxyprogesterone (*a*). *C*, ^{1}H NMR spectra of product (*b*) and 16α,17α-dihydroxyprogesterone (*a*) in $CDCl_3$ (600 MHz). Note that the C-18, C-19, and C-21 methyl signals are intact and the chemical shifts of the H-7 protons appear to be moved upfield, as predicted (Table 1). See text and Ref. 42 for discussion, and see supplemental Figs. S-1–S-4 for two-dimensional NMR spectra.

of its 17α-hydroxylated products). The new product, which was converted to its 3-keto-Δ^4 counterpart by cholesterol oxidase, co-chromatographed with standard 16α-hydroxyandrostenedione (Fig. 10). The 16-hydroxylation product of P450 17A1 was also observed when androstenedione was used as the substrate (Fig. 11). The ability of P450 17A1 to form 16-hydroxylated androgens is physiologically relevant in that estriol, an abundant and characteristic estrogen dur-

TABLE 1
NMR shift assignments for 6β,16α,17α-trihydroxyprogesterone



Carbon atom	δ_C	δ_H
1	36	2.06, 1.74 (COSY δ 2.53)
2	34.3	2.53, 2.42
3	205.1 (HMBC-2CH_2)	
4	127.7	5.84 (broad s)
5	167.5	
6	73.0	4.38 (broad s) (NOESY to δ 5.84)
7	38.5	1.98, 1.34 (COSY δ 4.38)
8	29.5 (43, 44)	1.99
9	52.9 (HMBC 19-CH_3, 1-H)	1.02
10	42.7	
	(HMBC – C19-CH_3/C6-CH/C2-CH_2)	
11	20 (43)	1.66 (COSY δ 1.08, 1.94), δ 1.48
12	30.8	1.97, 1.53
13	35.4 (HMBC 18-CH_3)	
14	48.9 (43, 44)	2.03 (W-coupling to δ 5.09, four bonds away)
15	33.6	1.59, 1.92 (COSY δ 5.09)
16	72.3	5.09
17	88.6	
18	15.1	0.79
19	19.5	1.40
20	211.4	
21	27.4	2.28

ing human pregnancies, arises from the aromatization of 16α-hydroxy androgens (70, 71).

The current working hypotheses we favor are shown in some detail in Fig. 19. Two involve an acetyl radical and one a steroid dioxetane intermediate, which are both considered viable entities.

B Ring Hydroxylation of 16α,17α-Dihydroxyprogesterone by P450 17A1—Surprisingly the 6β-position was hydroxylated when 16α,17α-dihydroxyprogesterone was used as a substrate for P450 17A1 (Fig. 15). This shift in regioselectively from the D-ring to the B-ring of the steroid by P450 17A1 was due to the presence of two hydroxyl groups on the 16α- and 17α-positions. Interestingly, regioselectivity was switched from the B-ring to the C–D-ring of the steroid in another P450 system when the Δ^4-double bond was reduced. P450 3A4 hydroxylates the 6β-position of testosterone (B-ring of the steroid); however, with 5α-dihydrotestosterone as the substrate, P450 3A4 oxygenated the 18β-methyl group (between the C–D-ring of the

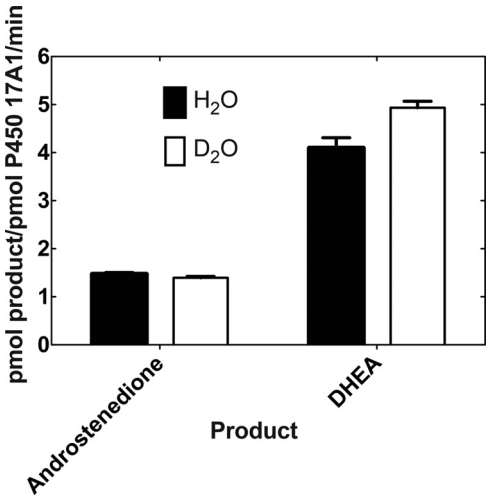


FIGURE 16. **Kinetic solvent isotope effects on 17α-hydroxyprogesterone and 17α-hydroxypregnenolone 17α,20-lyase reactions catalyzed by P450 17A1 (in the presence of NADPH-P450 reductase, NADPH, and cytochrome b_5).** Results are shown as means of four individual experiments ± S.D.

steroid) (72). The causes for the switch in regioselectivities of the different P450 systems are probably not the same. Moreover, P450 17A1, which normally hydroxylates the α-face of (the D-ring of) its steroid substrates (pregnenolone and progesterone), introduced a hydroxyl group on the β-face of 16α,17α-dihydroxyprogesterone. The hydroxylation of the opposite face can be rationalized from overlaying 17α-hydroxyprogesterone and 16α,17α-dihydroxyprogesterone (Fig. 20). When the C10, C14, and O16 atoms from 16α,17α-dihydroxyprogesterone were aligned with the C14, C10, and O3 atoms of 17α-hydroxyprogesterone, the O17 atom of 17α-hydroxyprogesterone was positioned 1.4 Å away from the C6 atom of 16α,17α-dihydroxyprogesterone, the site where the new oxygen atom is introduced. Additionally, the 17-oxygen of 17α-hydroxyprogesterone was directed at the β-face of 16α,17α-dihydroxyprogesterone. The 3-oxo group of 17α-hydroxyprogesterone has been shown to hydrogen bond to Asn-202 of P450 17A1 in the crystal structure (73). Based on our observations with 6β-hydroxylation of 16α,17α-dihydroxyprogesterone by P450 17A1 and the overlay of the two different substrates, we reason that the 16α-hydroxy group of 16α,17α-dihydroxyprogesterone hydrogen bonds to Asn-202 of P450 17A1, which in turn directs the 6β-hydrogen to the active iron center of the enzyme. Interestingly, the 17α,20-lyase product for the 16α,17α-dihydroxyprogesterone substrate (*i.e.* 16α-hydroxyandrostenedione) was detected by LC-MS analysis and co-elution with the standard, but this lyase product seems to be a minor product in comparison with the 6β-hydroxylation product.

17α,20-Lyase Reaction of 16α,17α-Dihydroxypregnenolone by P450 17A1—Conversely, when 16α,17α-dihydroxypregnenolone was used as the substrate for P450 17A1, the major product isolated from the incubation was 16α-hydroxy-DHEA, which is formed from the 17,20-carbon–carbon bond cleavage.

Oxygen Incorporation into 16α,17α-Dihydroxypregnenolone by P450 17A1—An additional oxygenation product (M + 16 of substrate) was detected by LC-HRMS when 16α,17α-dihydroxypregnenolone was used as the substrate. However, there was not enough purified material recovered to determine the

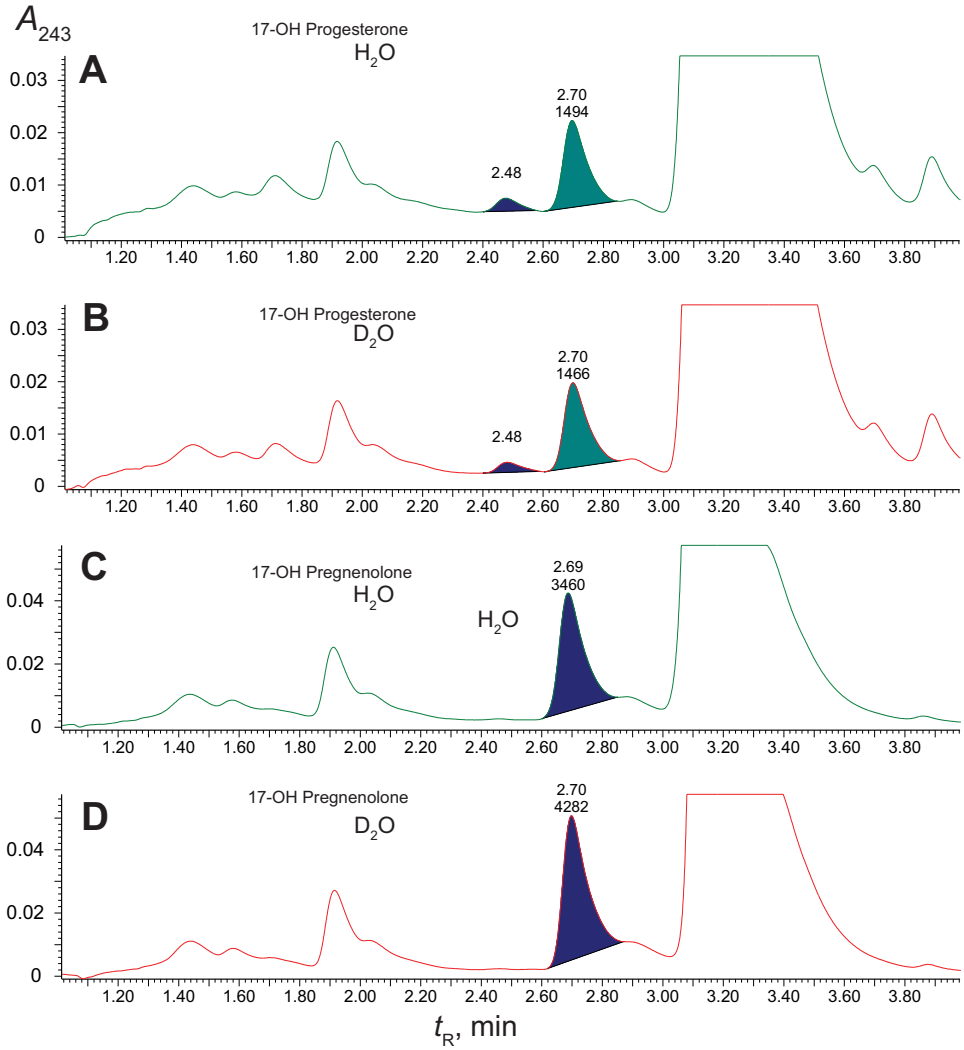


FIGURE 17. **Solvent kinetic deuterium isotope effects on 17α-hydroxyprogesterone and 17α-hydroxypregnenolone reactions catalyzed by P450 17A1 (in the presence of NADPH-P450 reductase and cytochrome b_5).** Retention times (t_R) and integration units are indicated on the chromatograms. The substrate concentration was 30 μM in all cases, and the reactions were done in either H_2O or 95% D_2O (v/v) at pH or pD 7.4. *A* and *B*, 17α-hydroxyprogesterone; *C* and *D*, 17α-hydroxypregnenolone. *A* and *C*, H_2O; *B* and *D*, 95% D_2O (v/v).

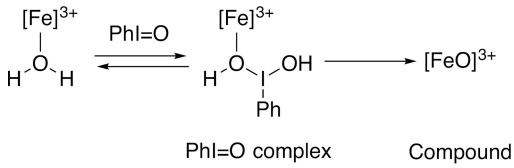


FIGURE 18. **Possible oxidizing alternative to compound I in the iodosylbenzene (*PhI=O*)-supported reactions (46).**

location of the oxygen on the steroid ring by ^{1}H NMR. A possible site of oxidation may be the C21-position. Alternatively, from the knowledge of 6β-hydroxylation reactivity of P450 17A1 with 16α,17α-dihydroxyprogesterone as the substrate, the oxygen may be incorporated in two other possible sites as follows: (i) the $\Delta^{5,6}$-double bond of the substrate to form the 5,6-epoxide or (ii) the C7-position may be hydroxylated. Epoxidation activity of P450 17A1 has been previously reported with a $\Delta^{16,17}$-steroid substrate (74). Nevertheless, the shift in favoring C–C bond cleavage reactivity over hydroxylation when using the 3β-hydroxy-Δ^5 substrate (16α,17α-dihydroxypregnenolone) instead of the 3-keto-Δ^4 substrate (16α,17α-dihydroxypregnenolone) is similar to what occurs with pregnenolone and progesterone (*i.e.* 17α,20-carbon,carbon bond cleavage *versus* 16α-hydroxylation). This observation may be related to the hydrogen bonding that occurs between the 3β-hydroxy group of the 3β-hydroxy-Δ^5 substrate and Asn-202 of the enzyme.

Kinetic Solvent Isotope Effects Do Not Support a Ferric Peroxide Mechanism—One argument against the proposed acetyl radical mechanism (Fig. 3*B*) is a reported *inverse* kinetic solvent deuterium isotope effect (0.39) reported by Sligar and co-workers (23). If the mechanism in Fig. 3, *B, C,* or *F, arrow b,* were valid, the abstraction of a hydrogen atom from the 17-hydroxyl group (Fig. 3*B*) or the heterolytic cleavage of an O–H bond (Fig. 3*D*) might be expected to be a (partially) rate-limiting step, and an inhibitory effect of hydroxyl deuteration might be expected. In contrast, a similar study by Swinney and Mak (35) reported that (30%) D_2O *attenuated* androgen formation from 17α-hydroxyprogesterone using microsomes from pig testes as the enzyme source (k_H/k_D ~1.25 at pH 7), suggesting that the 17α,20-lyase reaction is dependent on compound I formation either through the pro-

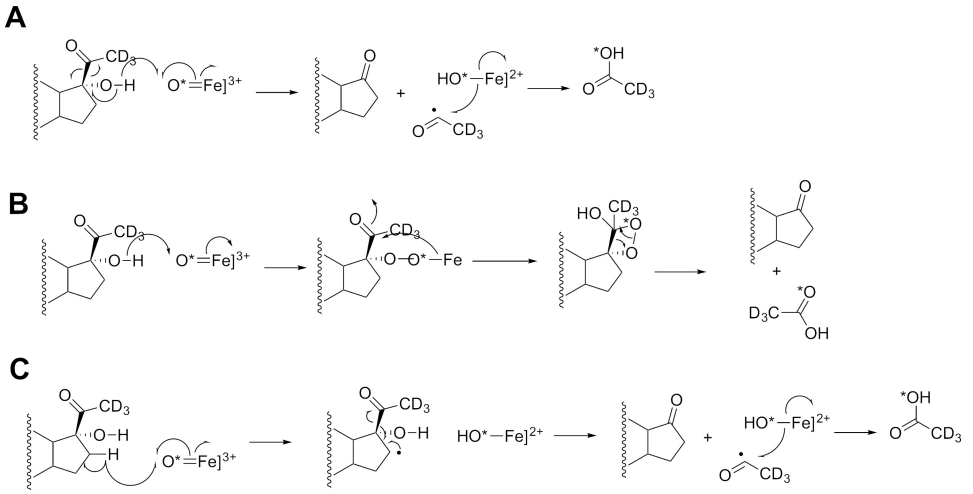


FIGURE 19. **Mechanisms of P450 17A1-catalyzed 17α,20-lyase reaction consistent with ^{18}O labeling (7), oxygen surrogate results, and solvent kinetic isotope results.** The course of an ^{18}O label (from $^{18}O_2$) is indicated with an *asterisk* (7, 40). *A*, compound I mechanism with hydrogen atom abstraction from the 17α alcohol followed by C17-C20 bond scission to yield an acetyl radical; *B*, addition of the 17α hydroxyl group to compound I to yield an iron peroxide-C17 complex, followed by decomposition via a C17-C20 dioxetane; *C*, compound I mechanism with hydrogen atom abstraction from the C16 carbon. See text for discussion and also Fig. 3.

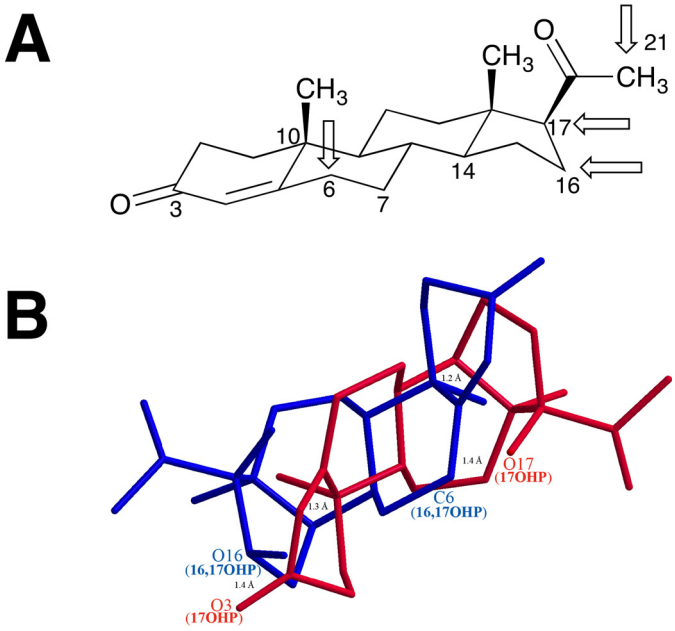


FIGURE 20. **Sites of hydroxylation of progesterone by P450 17A1.** *A*, chair configuration of progesterone, with the four sites of attack indicated by *arrows*. *B*, wire diagram of 17α-hydroxyprogesterone (*17OHP*, *red*) and 16α,17α-dihydroxyprogesterone (*16,17OHP*, *blue*) overlaid, with the latter in an alternative configuration to show the proximity of the C-6 atom of 16α,17α-hydroxyprogesterone with the 17-hydroxy group of 17α-hydroxyprogesterone. The model was made using Chem3D, with a minimum root mean square error of 0.1 and minimum root mean square gradient of 0.01. The C14, C10, and O3 atoms of 17α-hydroxyprogesterone were aligned with the C10, C14, and O16 atoms of 16α,17α-dihydroxyprogesterone, respectively, by displaying the distance measurements of each pair of atoms and then running an overlay minimization calculation. The *green lines* indicate the pair of atoms that were aligned (after overlay minimization, the distances between C14 of 17-OHP and C10 of 16,17-OHP; C10 of 17-OHP and C14 of 16,17-OHP; and O3 of 17-OHP and O17 of 16,17-OHP were 1.2, 1.3, and 1.4 Å, respectively, and are shown as *green lines*). The distance between the O17 atom of 17α-hydroxyprogesterone and C6 atom of 16α,17α-dihydroxyprogesterone was 1.4 Å.

tonation of the distal oxygen of ferric peroxide (*cf.* P450 catalytic cycle) or deuterium atom abstraction from the 17-hydroxy group of the substrate (Fig. 3*B*).

Because of the discrepancy, we reinvestigated the results in our own system (Fig. 16). Running the normal P450 17A1 reaction (with NADPH-P450 reductase and cytochrome b_5) in 95% D_2O showed no significant change in the rate of conversion of 17α-hydroxyprogesterone to androstenedione and a small but statistically significant change in the rate of oxidation of 17α-hydroxypregnenolone to DHEA, with an apparent isotope effect of 0.83 (Fig. 17), which is much less than the effect (0.39) reported by Gregory *et al.* (23). Interpretation of solvent kinetic deuterium isotope effects is complex (75), in that protonation and deprotonation can occur throughout the amino acid side chains of an enzyme, not only on an iron-oxygen complex. The reason for the small inverse isotope effect with one lyase substrate but not another (Figs. 16 and 17) is unclear. The opposite pattern between the solvent isotope effects for the 17α-hydroxypregnenolone lyase and the 16-hydroxylation reactions is qualitatively consistent with the report of Gregory *et al.* (23). One possibility is that the Δ^5 substrate (17α-hydroxypregnenolone) 3-hydroxy group exchanges with deuterium and that this has an effect on the juxtaposition of the substrate in the active site. The hydroxyl moiety has been shown by Scott and coworkers (73, 76) to be in hydrogen bonding distance to Asn-202 of human P450 17A1. A substitution of the 3-hydroxyl group by deuteration (*i.e.* −OD) could shift the substrate to favor the lyase reaction *versus* 16-hydroxylation. However, the lack of solvent isotope effects does not allow any definite conclusions about the rate-limiting nature of the abstraction of a proton or hydrogen atom from the 17-OH group, due to the multiple complex influences from solvent deuterium on enzyme function.

Although resonance Raman spectra of what is reported to be the human P450 17A1 FeO_2^- complex have recently been published (9, 25), two caveats are as follows: (i) no cytochrome b_5 (for which the 17α,20-lyase reaction is very dependent, *e.g.* Fig. 8*B*) was present, and (ii) the observed complex was not tested for its catalytic competence, *i.e.* to form product(s). Even if the FeO_2^- complex did form the normal products (androstenedione and DHEA, plus the 16-hydrox-

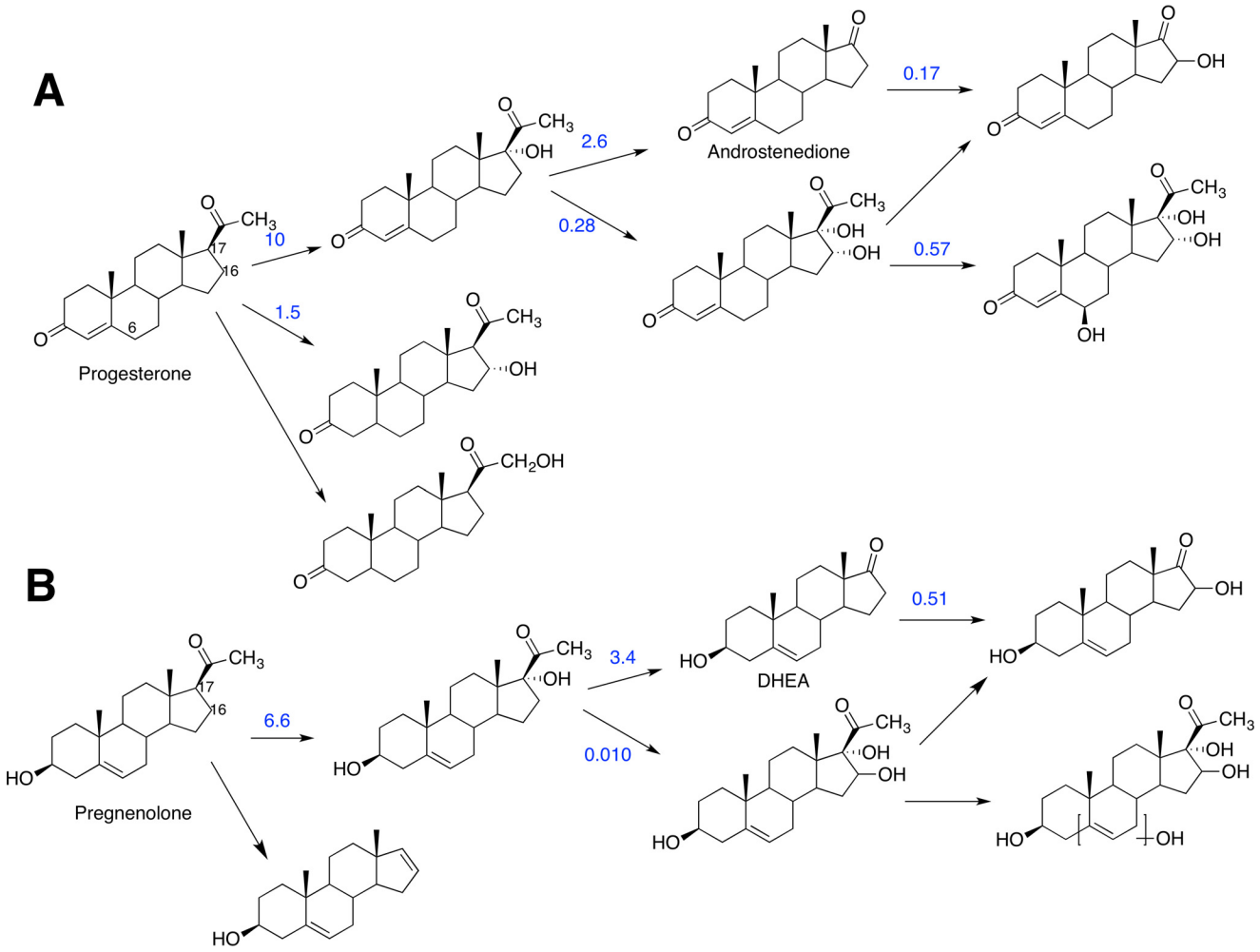


FIGURE 21. **Summary of current known reactions of human P450 17A1.** See also Refs. 7, 26, 53. Rates determined at high substrate concentrations (approximating k_{cat} conditions) in this study are indicated, in units of nanomoles of product formed/min/nmol P450 17A1, when available. *A*, products formed from progesterone; *B*, products formed from pregnenolone.

ylation products, which is unlikely) in these experiments, the simultaneous or subsequent intermediacy of an FeO^{3+} species as well could not be ruled out.

Conclusions—The ability of iodosylbenzene, but not H_2O_2, to support the lyase reaction provides what may be the strongest evidence in favor of a compound I mechanism, in that iodosylbenzene cannot possibly form a peroxy intermediate. The apparent rate of the lyase reaction was similar to that of the NADPH-supported reaction (without cytochrome b_5) in the case of the 17α-hydroxyprogesterone reaction and was somewhat less than that of the NADPH-supported reaction (without cytochrome b_5) in the case of the 17α-hydroxypregnenolone lyase reaction (Figs. 8 and 9). Ideally, the compound I form of P450 17A1 could be prepared using the approaches that Green and co-workers (8, 55, 64, 77) have used with two bacterial P450s (64), and the reaction could be investigated directly. Nevertheless, in considering all of the literature in this field and that presented here in this article, the iodosylbenzene and H_2O_2 results (Fig. 10) are difficult to dismiss, even if they are not physiological, and are interpreted as evidence for a compound I reaction (Fig. 19).

The multiple hydroxylations are probably catalyzed by FeO^{3+} intermediates, formed with P450 17A1-17α-hydroxy steroid complexes. It is possible that individual reactions (*i.e.* hydroxylation, lyase) proceed from different FeO complexes, although it is simpler to explain all as emanating from a single iron-oxygen intermediate. The myriad of reactions is depicted in Fig. 21 and reveals a surprising flexibility in the P450 17A1 enzymes. As indicated, P450 17A1 has been shown to catalyze 21-hydroxylation of progesterone (53). Our observed rates are indicated in the figure. Lyase reactions are not overly dominant. The biological activities of most of the products are, to our knowledge, still unknown.

In summary, we have provided evidence that a compound I-type mechanism (Fig. 19) can be involved in the 17α,20-lyase reactions. Our results do not rule out a ferric peroxide mechanism, nor do they define the fraction of the normal reaction that is catalyzed by each of the two mechanisms, if both are operative. If further research implicates compound I in this reaction, then few strong cases for P450 ferric peroxide chemistry will remain, at least in the field of steroid metabolism (33, 64, 78).

Experimental Procedures

General—Bruker instruments (400 and 600 MHz) were used to acquire NMR spectra in the Vanderbilt facility. CD_3CN and

$CDCl_3$ residual proton peaks were referenced to δ 1.94 and 7.26 ppm, respectively, and the $CDCl_3$ triplet in the carbon spectrum was referenced to δ 77.16 ppm and CD_3CN was referenced to 118.26 ppm (79). Unless specified otherwise, all chemicals were purchased from Sigma-Aldrich. A modified version of the nitrosourea reagent was synthesized according to Ref. 33 using 2-(2-pyridyl)ethylamine (instead of 3-(3-pyridyl)propylamine) as the starting material, as described in detail here.

Reagents—17α-Hydroxy-[21,21,21-$^2H_3$]progesterone (96.5% atomic excess as judged by ^1H NMR, 97.9% atomic excess as judged by LC-MS) was synthesized and characterized as described previously (53). 17α-Hydroxy-[21,21,21-$^2H_3$] pregnenolone (nominal 98.4% atomic excess) and 17α-hydroxy-[2,2,4,6,6,21,21,21-$^2H_8$]progesterone (nominal 98.7% atomic excess) were purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). 16α,17α-Dihydroxyprogesterone (algestone) was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). 16α-Hydroxyandrostenedione was obtained from Steraloids (Newport, RI).

Enzymes—Recombinant human P450 3A4 with a C-terminal His_5 tag was expressed in *Escherichia coli* and purified as described previously (80, 81). *E. coli* recombinant rat NADPH-P450 reductase and human liver cytochrome b_5 were prepared as described by Hanna *et al.* (82) and Guengerich (83), respectively.

Recombinant human P450 17A1 (with a C-terminal His_4 tag) was expressed in *E. coli* and purified by metal-affinity chromatography using a protocol adapted from those previously reported (76, 84, 85). Briefly, an *E. coli* codon-optimized cDNA, corresponding to the amino acid sequence reported by DeVore and Scott (76), was purchased (Genewiz, South Plainfield, NJ) and inserted into the pCW-Ori(+) expression vector. The construct was used to transform competent *E. coli* JM109 cells (Agilent), and an isolated colony was used to inoculate 100 ml of Luria-Bertani medium (containing 100 μg/ml ampicillin), which was then incubated at 37 °C with shaking at 250 rpm overnight (12–14 h). Expression ensued by inoculating 1 liter of Terrific Broth medium, containing 100 mg/liter ampicillin and 250 μl/liter of trace elements (86), with 10 ml of the overnight culture and incubating at 37 °C (250 rpm) for ~4 h (OD_{600} ~0.32). The expression culture was then supplemented with 1 mM isopropyl β-D-1-thiogalactopyranoside and 1 mM δ-aminolevulinic acid, and the incubation conditions were changed to 30 °C and 200 rpm. After ~40 h, the culture was centrifuged at 5000 × *g* for 10 min, and the bacterial pellet was resuspended in 300 ml of 100 mM Tris-HCl buffer (pH 7.6) containing 500 mM sucrose and 0.5 mM EDTA and placed on ice. The suspension was then treated with 60 μl of a 50 mg/ml lysozyme solution/g of bacterial pellet and incubated on ice for 30 min, with gentle mixing every 10 min. All subsequent steps were conducted on ice or at 4 °C. Next, a spheroplast pellet was obtained by centrifugation at 5000 × *g* for 10 min and resuspended in 25 ml of 300 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol (v/v), 6 mM $Mg(CH_3CO_2)_2$, 0.1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture (cOmplete™, EDTA-free, Roche Applied Science). The spheroplasts were lysed by sonication, and debris and unbroken cells were removed by centrifugation at 9000 × *g* for 20 min. The cytosol was cleared of the membrane fraction by centrifugation at 100,000 × *g* for 60 min and was supplemented with 300 mM NaCl and 20 mM imidazole prior to loading onto a nickel-nitrilotriacetic acid resin (Qiagen) bed that had been equilibrated with 300 mM potassium phosphate buffer (pH 7.4) containing 300 mM NaCl, 20% glycerol (v/v), 20 mM imidazole, and 0.1 mM DTT. The bound protein was washed with 10 bed volumes of the same buffer and eluted with the same buffer containing 250 mM imidazole. The purified enzyme was then dialyzed four times against 100-fold volumes of 200 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol (v/v), 0.1 mM EDTA, and 0.1 mM DTT and stored at −70 °C until use.

Chemical Synthesis

(2-(Pyridin-2-yl)ethyl)urea (33)—A solution of 2-(2-pyridyl) ethylamine (0.753 g, 6.17 mmol) and benzotriazole-1-carboxamide (1.00 g, 6.17 mmol) in tetrahydrofuran was heated under reflux for 12 h. The reaction mixture was concentrated *in vacuo* and purified by flash column chromatography (gradient of hexanes to 50% hexanes in ethyl acetate (v/v) to 10% CH_3OH in CH_2Cl_2 (v/v)) to afford (2-(pyridin-2-yl)ethyl)urea as a white solid (1.00 g, 6.06 mmol, 98%). R_f 0.28 (silica gel GF, CH_2Cl_2/CH_3OH, 9:1, v/v); ^1H NMR (600 MHz, CD_3CN) δ 8.50 (d, J = 4.8 Hz, 1H), 7.66 (ddd, J = 7.7, 7.6, 2.0 Hz, 1H), 7.21 (d, J = 7.9 Hz, 1H), 7.17 (dd, J = 7.9, 4.8 Hz, 1H), 5.25 (br s, 1H), 4.54 (br s, 2H), 3.44 (d, J = 6.7 Hz, 1H), 3.42 (d, J = 6.7 Hz, 1H), 2.89 (apparent t, J = 6.8 Hz, 2H); ^{13}C NMR (150 MHz, CD_3CN): δ 160.8, 159.4, 150.1, 137.3, 124.2, 122.3, 40.3, 38.9.

1-Nitroso-1-(2-(pyridin-2-yl)ethyl)urea (33)—$NaNO_2$ (0.40 g, 6.1 mmol) was added to a solution of (2-(pyridin-2-yl)ethyl) urea (1.0 g, 6.1 mmol) in 50 ml of 1.2 M aqueous HCl at 0 °C. After 2 h, the reaction was diluted with CH_2Cl_2 (100 ml), and the resulting mixture was washed with $NaHCO_3$ (saturated aqueous solution, 2× 50 ml). The organic layer was dried with $MgSO_4$ and concentrated under a stream of nitrogen to afford the nitrosourea (0.45 g, 2.3 mmol, 38%) as a yellow oil, which was used without further purification as the precursor to generate the diazo reagent for derivatization of the acetic acid product of P450 17A1. R_f 0.42 (silica gel GF, CH_2Cl_2/CH_3OH, 9:1, v/v); ^1H NMR (600 MHz, CD_3CN) δ 8.53 (d, J = 4.8 Hz, 1H), 7.70 (ddd, J = 7.7, 7.6, 1.8 Hz, 1H), 7.29 (d, J = 7.7 Hz, 1H), 7.22 (dd, J = 7.7, 4.7 Hz, 1H), 3.71 (apparent t, J = 6.6 Hz, 2H), 3.05 (apparent t, J = 6.6 Hz, 1H).

2-(Pyridin-2-yl)ethyl Acetate—2-(Pyridin-2-yl)ethanol (1.0 ml, 8.9 mmol) and acetic anhydride (840 μl, 8.9 mmol) were stirred overnight in a 4-ml screw cap vial. The reaction was dissolved in CH_2Cl_2 and treated with aqueous $NaHCO_3$ to remove the by-product CH_3CO_2H, and the product was purified by preparative TLC (silica gel GF, 20 × 20 cm, 500 μm) using hexane/ethyl acetate (1:1, v/v) as the mobile phase. The product was scraped from the TLC plate, extracted with CH_2Cl_2, and filtered. The solvent was evaporated under a stream of nitrogen to obtain 2-(pyridin-2-yl)ethyl acetate as a pale yellow liquid (980 mg, yield 67%). R_f 0.61 (silica gel GF, CH_2Cl_2/CH_3OH, 9:1, v/v); ^1H NMR (400 MHz, $CDCl_3$): δ 8.55 (d, J = 4.6 Hz, 1H), 7.61 (ddd, J = 7.7, 7.6, 1.8 Hz, 1H), 7.18 (d, J = 7.8 Hz, 1H), 7.14 (dd, J = 7.6, 5.0 Hz, 1H), 4.46 (t, J = 6.7 Hz, 2H), 3.12 (t, J = 6.7 Hz, 2H), 2.02 (s, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$): 171.1, 158.1, 149.5, 136.5, 123.5, 121.7, 63.7, 37.4, 21.0;

HRMS (ESI^+): calculated for $C_9H_{11}NO_2$ $[M + H]^+$ *m/z* 166.08626, found 166.08678 (Δ 3.1 ppm).

16α,17α-Dihydroxypregnenolone—16α,17α-Dihydroxypregnenolone was synthesized in two steps from commercially available 16,17-dehydropregnenolone-3-acetate. 16,17-Dehydropregnenolone-3-acetate was converted to 16,17-dehydropregnenolone according to a previously reported procedure (74). 16,17-Dehydropregnenolone was subjected to dihydroxylation conditions loosely based on a procedure that was previously reported (87); $KMnO_4$ (105 mg, 0.67 μmol) in acetone/H_2O (6:1, v/v, 70 ml) was added over 10 min with an addition funnel to 16,17-dehydropregnenolone (209 mg, 0.67 μmol) in acetone (50 ml) and HCO_2H (50 μl) at 0 °C. After the addition was complete, the reaction mixture was stirred for 2 min and then washed with H_2O (100 ml) and extracted with ethyl acetate (200 ml). The organic layer was concentrated *in vacuo* and purified by flash column chromatography (100% hexanes to 50% ethyl acetate/hexanes, v/v), and the most pure fraction (as judged by TLC analysis) was concentrated (10 mg, white solid). The white solid was further purified using an HPLC-UV system with a Beckman Ultrasphere octadecylsilane column (10 × 250 mm, 5 μm) with the following H_2O and CH_3OH gradients at a flow rate of 4 ml/min: 0–2.3 min, 77% CH_3OH; 2.3–11.3 min, linear gradient from 77 to 88% CH_3OH; 11.3–11.6 min, hold at 88% CH_3OH; 11.6–12.4 min, linear gradient from 88 to 77% CH_3OH; 12.4–15 min, hold at 77% CH_3OH (all v/v). Elution of the steroid was detected at 215 nm. The CH_3OH from the collected fraction was evaporated under a N_2 stream, and H_2O was removed by lyophilization. The purified material was used for incubation with P450 17A1. R_f 0.3 (silica gel GF, ethyl acetate/hexanes, 1:1, v/v); ^1H NMR (600 MHz, $CDCl_3$): δ 5.34–5.30 (m, 1H), 5.08–5.05 (m, 1H), 3.79 (broad s, 1H), 3.56–3.51 (m, 1H), 2.32–2.28 (m, 1H), 2.25 (s, 3H), 2.24–2.21 (m, 1H), 2.07–1.92 (m, 3H), 1.88–1.77 (m, 3H), 1.65–1.58 (m, 2H), 1.53–1.44 (m, 6H), 1.00 (s, 3H), 0.70 (s, 3H).

Iodosylbenzene—Iodosylbenzene was freshly prepared by NaOH hydrolysis of iodobenzene diacetate in 75% yield (88) and stored at −20 °C.

Assays

$^{18}O_2$ Incubations—The standard reconstituted P450 17A1 system contained P450 17A1 (8.4 μM), NADPH-P450 reductase (13 μM), cytochrome b_5 (14 μM), 130 μM 17α-hydroxy-[21,21,21-$^2H_3$]pregnenolone or 100 μM 17α-hydroxy-[2,2,4,6,6,21,21,21-$^2H_8$]progesterone, and L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine (80 μM) in 2.2-ml incubation mixtures containing 50 mM potassium phosphate buffer (pH 7.4). Reaction mixtures were placed in Thunberg tubes, and air was removed on a gas train equipped with a manifold (89, 90) (three exchanges of argon/vacuum, 5 min each cycle). After introduction of $^{18}O_2$ (Sigma-Aldrich, 99% atomic excess, pressurized cylinder) into a Thunberg tube under vacuum, each reaction was initiated by adding an NADPH-generating system (10 mM glucose 6-phosphate, 0.5 mM $NADP^+$, and 2 μg/ml yeast glucose-6-phosphate dehydrogenase (91); 8% of total reaction volume) tipped from the stopper reservoir, with mixing. Incubations were conducted in a water bath at 37 °C for 30 min for 17α-hydroxy-[21,21,21-$^2H_3$]pregnenolone substrate or 60 min for 17α-hydroxy-[2,2,4,6,6,21,21,21-$^2H_8$]progesterone substrate with shaking at 100 rpm.

The reactions were quenched with CH_2Cl_2 (5 ml), and 500 μl of 3 M HCl (chilled to 0 °C) for the 17α-hydroxy-[21,21,21-$^2H_3$]pregnenolone substrate or 1 ml of 3 M HCl (chilled to 0 °C) for the 17α-hydroxy-[2,2,4,6,6,21,21,21-$^2H_8$]progesterone substrate was added to decrease the pH to ~1 and facilitate extraction of acetic acid into the organic layer. For cases with 17α-hydroxy-[2,2,4,6,6,21,21,21-$^2H_8$]progesterone as the substrate, the mixture (after addition of HCl) was mixed with a vortex device and centrifuged at 1500 × *g* for 1 min to remove the emulsion. Before the reaction of the product acetic acid with the diazo reagent, the organic extracts were collected, and residual water was removed using anhydrous $MgSO_4$ (~50 mg for each extraction).

Derivatization of Acetic Acid—Diazoethylpyridine was prepared *ex tempore* from 1-nitroso-1-(2-(pyridin-2-yl)ethyl)urea (5 mg) in diethyl ether (2 ml), after treatment with KOH (1 ml of a 30% (w/v) solution in H_2O) (33). The organic layer (containing the diazo reagent) was dried with anhydrous $MgSO_4$ (~50 mg), filtered with a cotton-plugged Pasteur pipette, and reacted with an organic extract of each P450 17A1-steroid-$^{18}O_2$ incubation. The solvent was evaporated under a stream of nitrogen, and residues were dissolved in CH_3CN (70 μl).

LC-MS Analysis—LC-MS analysis of deuterium-labeled 2-(pyridin-2-yl)ethyl acetate from $^{18}O_2$ incubations was performed using an Acquity UPLC system connected to a Thermo LTQ XL Orbitrap mass spectrometer operating in the electrospray ionization (ESI) positive ion mode. A Phenomenex Kinetex® 2.6-μm C8 100 Å, LC column (100 × 2.1 mm) was used for separation of the acetic acid derivative at a flow rate of 0.3 ml/min with the following gradient: 0–1.0 min, 100% A (v/v); 4.0–5.2 min, 100% B (v/v); 5.3–8.0 min, 100% A (v/v); mobile phase A was 10 mM NH_4HCO_2 in H_2O (v/v); and mobile phase B was 10 mM NH_4HCO_2 in 95:5 CH_3CN/H_2O (v/v).

For the $^{18}O_2$ incubation assays, the LTQ mass spectrometer was tuned in the electrospray ionization positive mode using synthetic 2-(pyridin-2-yl)ethyl acetate (see above). The tune settings were as follows: sheath gas flow rate, 15 (arbitrary units); auxiliary gas flow rate, 5 (arbitrary units); sweep gas flow rate, 0 (arbitrary units); spray voltage, 4 kV; capillary temperature, 300 °C; capillary voltage, 16 V; tube lens, 30 V.

The LTQ Orbitrap XL high resolution mass spectrometer was calibrated with the ESI-positive ion calibration solution by direct infusion (10 μl/min with a 500-μl Hamilton syringe) as done previously (33). The mass spectrometer was first tuned to the standard solution with *m/z* 524.3 (methionine/arginine/phenylalanine/alanine acetate), and the tube lens voltage was set to 145 V to fragment caffeine (*m/z* 195 to 138).

17α-Hydroxysteroid Reactions with Oxygen Surrogates—The standard reconstituted system used for comparison included P450 17A1 (0.5 μM for iodosylbenzene, 0.1 μM for H_2O_2), NADPH-P450 reductase (2.0 μM), cytochrome b_5 (0.5 μM), and L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine (10 μM) in 0.5-ml incubation mixtures containing 50 mM potassium phosphate buffer (pH 7.4) and the NADPH-generating system. Assays were done as in the case of the ^{18}O-labeling work (see above) except that the incubations were aerobic, as described previously (37), using 10 μM 17α-hydroxyprogesterone or 17α-

hydroxypregnenolone with P450 17A1 (0.5 μM) and no reductase, in the absence or presence of cytochrome b_5 (0.5 μM). H_2O_2 (to 10 mM) or iodosylbenzene (to 2 mM) was added at varying concentrations (from aqueous stocks). The incubations were done for 5 min with H_2O_2 and for 30 s with iodosylbenzene (41, 92), with extraction into CH_2Cl_2 and analysis of the conversion of 17α-hydroxyprogesterone to androstenedione and of 17α-hydroxypregnenolone to DHEA by UPLC. The Δ^5 steroids were converted to Δ^4 steroids by treatment with cholesterol oxidase prior to LC-UV analysis (37). Iodosylbenzene reactions were conducted for a short time period because the reagent is very destructive to P450 heme (41).

Product analysis was done on a Waters Acquity UPLC system with a Waters Acquity UPLC Ethylene Bridged Hybrid (BEH) octadecylsilane (C_{18}) column (2.1 × 100 mm, 1.7 μm). LC conditions were as follows: solvent A consisted of 70% CH_3OH and 30% H_2O (v/v), and solvent B was 100% CH_3CN. The products were resolved by a 0.2 ml min^{-1} gradient with the following steps: 0–1 min, hold at 5% B (v/v), 1–4 min, linear gradient from 5 to 30% B (v/v); 4–4.5 min, linear gradient from 30 to 40% B (v/v); 4.5–4.55 min, 40 to 95% B (v/v); 4.55–6.75 min, hold at 95% B (v/v); 6.75–7 min, 95 to 5% B (v/v); and 7–10 min, hold at 95% B (v/v). The column temperature was maintained at 40 °C, and the Δ^4 steroids were quantified by their absorbance at 243 nm.

LC-UV-MS Analysis of New Steroid Products—An LTQ Orbitrap XL mass spectrometer was tuned in the atmospheric pressure chemical ionization-positive mode with commercially available steroid solutions in a 1:1 mixture (v/v) of H_2O and CH_3CN (16α-hydroxyandrostenedione and 16α,17α-dihydroxyprogesterone). The tune settings were as follows: vaporizer temperature, 350 °C; sheath gas flow rate, 50 (arbitrary units); auxiliary gas flow rate, 5 (arbitrary units); sweep gas flow rate, 0 (arbitrary units); discharge current, 10 μA; capillary temperature, 275 °C; capillary voltage, 10 V; tube lens, 25 V. The same LC method used for UV analysis (see above) was employed for the Δ^4 steroid products. The LC conditions for the Δ^5 steroids were as follows: solvent A consisted of 95% H_2O and 5% CH_3OH (v/v), and solvent B was 95% CH_3OH and 5% H_2O (v/v). The gradient steps were as follows: 0–1.5 min, hold at 60% B (v/v); 1.5–7.5 min, linear gradient from 60 to 85% B (v/v); 7.5–7.75 min, hold at 85% B (v/v); 7.75–8.25 min, 85 to 60% B (v/v); and 8.25–10 min, hold at 60% B (v/v). The column was kept at ambient temperature.

Solvent Kinetic Isotope Effect Assays—These assays were also done as in the case of the oxygen surrogate experiments (see above), with the incubations done aerobically as described previously (37), using 30 μM 17α-hydroxyprogesterone or 17α-hydroxypregnenolone with P450 17A1 (0.5 μM)/cytochrome b_5 (0.5 μM); NADPH-P450 reductase (2 μM); and L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine (10 μM) in 0.5 ml incubation mixtures containing 50 mM potassium phosphate buffer (pH 7.4), with 1 mM NADPH. In the D_2O experiments, the content of D_2O was 95% (v/v), with the pD adjusted (pH = pD + 0.4) (75, 93). Incubations were for 60 s at 37 °C, and the products were analyzed as for the oxygen surrogate experiments (see above).

Isolation of 6β,16α,17α-Trihydroxyprogesterone as a Product—A 20-ml reaction mixture consisting of the same components described in the oxygen surrogate experiments (see above), using 280 units/ml of catalase and 40 μM 16α,17α-dihydroxyprogesterone, was run overnight (~20 h). The product was extracted from the aqueous mixture with 200 ml of CH_2Cl_2, and the solvent was evaporated. The dried product was purified using the same system outlined for the 16,17-dihydroxypregnenolone purification (see above) using an isocratic HPLC method (63.5% CH_3OH) and peak detection at 243 nm.

Isolation of 16α-Hydroxy-DHEA as a Product—The same procedure described for the isolation of the 6β,16α,17α-trihydroxyprogesterone product (see above) was used, with the following exceptions. The enzyme concentrations were increased to 1 μM P450 17A1, 4 μM NADPH-P450 reductase, 1 μM cytochrome b_5, and 3700 units/ml catalase, with 50 μM 16α,17α-dihydroxypregnenolone and the NADPH-generating system. The incubation was run for 4 h. Purification was conducted using the same procedure detailed in the LC-UV purification of 16α,17α-dihydroxyprogesterone (see above), except that the wavelength used to detect the Δ^5 product was 215 nm.

Author Contributions—F. K. Y. synthesized most of the chemicals, did all of the NMR analysis, and did the ^{18}O analyses. E. G. purified the P450 17A1 and did most of the incubations and HPLC analyses, including some of the mass spectra. R. J. A. supervised part of the work and helped write the paper. F. P. G. oversaw the project, synthesized iodosylbenzene, and assembled the paper. All authors contributed to the writing of the paper and the conclusions.

Acknowledgments—We thank L. D. Nagy and T. T. N. Phan for preparing NADPH-P450 reductase; M. Bojić for preparing 2-(pyridine-2-yl)ethyl acetate and performing some of the preliminary experiments; K. M. Johnson for recording the NMR spectra of 2-(pyridine-2-yl)ethyl acetate; and K. Trisler for assistance in preparation of the manuscript.

Note Added in Proof—In the version of this article that was published as a Paper in Press on June 23, 2016, the spectra in Fig. 14*B* were inadvertently duplicated from Fig. 15*C*. This error has now been corrected and does not affect the results or conclusions of this work.

References

1. Rendic, S., and Guengerich, F. P. (2015) Survey of human oxidoreductases and cytochrome P450 enzymes involved in the metabolism of xenobiotic and natural chemicals. *Chem. Res. Toxicol.* **28,** 38–42
2. Guengerich, F. P. (2001) Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. *Chem. Res. Toxicol.* **14,** 611–650
3. Isin, E. M., and Guengerich, F. P. (2007) Complex reactions catalyzed by cytochrome P450 enzymes. *Biochim. Biophys. Acta* **1770,** 314–329
4. Ortiz de Montellano, P. R. (2015) in *Cytochrome P450: Structure, Mechanism, and Biochemistry* (Ortiz de Montellano, P. R., ed) 4th Ed., pp 111–176, Springer, New York
5. Miller, W. L., and Auchus, R. J. (2011) The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocr. Rev.* **32,** 81–151
6. Guengerich, F. P. (2015) in *Cytochrome P450: Structure, Mechanism, and Biochemistry* (Ortiz de Montellano, P. R., ed) 4th Ed., pp 523–785, Springer, New York
7. Yoshimoto, F. K., and Auchus, R. J. (2015) The diverse chemistry of cytochrome P450 17A1 (P450c17, CYP17A1). *J. Steroid Biochem. Mol. Biol.* **151,** 52–65
8. Krest, C. M., Onderko, E. L., Yosca, T. H., Calixto, J. C., Karp, R. F., Livada, J., Rittle, J., and Green, M. T. (2013) Reactive intermediates in cytochrome P450 catalysis. *J. Biol. Chem.* **288,** 17074–17081

9. Mak, P. J., Gregory, M. C., Denisov, I. G., Sligar, S. G., and Kincaid, J. R. (2015) Unveiling the crucial intermediates in androgen production. *Proc. Natl. Acad. Sci. U.S.A.* **112,** 15856–15861
10. Groves, J. T. (2006) High-valent iron in chemical and biological oxidations. *J. Inorg. Biochem.* **100,** 434–447
11. Bell, S. R., and Groves, J. T. (2009) A highly reactive P450 model compound I. *J. Am. Chem. Soc.* **131,** 9640–9641
12. Morand, P., Williamson, D. G., Layne, D. S., Lompa-Krzymien, L., and Salvador, J. (1975) Conversion of an androgen epoxide into 17β-estradiol by human placental microsomes. *Biochemistry* **14,** 635–638
13. Fishman, J. (1982) Biochemical mechanism of aromatization. *Cancer Res.* **42,** 3277s–3280s
14. Akhtar, M., Corina, D., Pratt, J., and Smith, T. (1976) Studies on the removal of C-19 in oestrogen biosynthesis using $^{18}O_2$. *J. Chem. Soc., Chem. Commun.*, **1976,** 854–856
15. Akhtar, M., Calder, M. R., Corina, D. L., and Wright, J. N. (1982) Mechanistic studies on C-19 demethylation in oestrogen biosynthesis. *Biochem. J.* **201,** 569–580
16. Goto, J., and Fishman, J. (1977) Participation of a nonenzymatic transformation in the biosynthesis of estrogens from androgens. *Science* **195,** 80–81
17. Hahn, E. F., and Fishman, J. (1984) Immunological probe of estrogen biosynthesis. Evidence for the 2β-hydroxylative pathway in aromatization of androgens. *J. Biol. Chem.* **259,** 1689–1694
18. Caspi, E., Wicha, J., Arunachalam, T., Nelson, P., and Spiteller, G. (1984) Estrogen biosynthesis: concerning the obligatory intermediacy of 2β-hydroxy-10β-formyl androst-4-ene-3,17-dione. *J. Am. Chem. Soc.* 1984, **106,** 7282–7283
19. Hackett, J. C., Brueggemeier, R. W., and Hadad, C. M. (2005) The final catalytic step of cytochrome P450 aromatase: a density functional theory study. *J. Am. Chem. Soc.* **127,** 5224–5237
20. Sen, K., and Hackett, J. C. (2012) Coupled electron transfer and proton hopping in the final step of CYP19-catalyzed androgen aromatization. *Biochemistry* **51,** 3039–3049
21. Mak, P. J., Luthra, A., Sligar, S. G., and Kincaid, J. R. (2014) Resonance Raman spectroscopy of the oxygenated intermediates of human CYP19A1 implicates a compound I intermediate in the final lyase step. *J. Am. Chem. Soc.* **136,** 4825–4828
22. Cole, P. A., and Robinson, C. H. (1990) Mechanism and inhibition of cytochrome P-450 aromatase. *J. Med. Chem.* **33,** 2933–2942
23. Gregory, M. C., Denisov, I. G., Grinkova, Y. V., Khatri, Y., and Sligar, S. G. (2013) Kinetic solvent isotope effect in human P450 CYP17A1-mediated androgen formation: evidence for a reactive peroxoanion intermediate. *J. Am. Chem. Soc.* **135,** 16245–16247
24. Khatri, Y., Gregory, M. C., Grinkova, Y. V., Denisov, I. G., and Sligar, S. G. (2014) Active site proton delivery and the lyase activity of human CYP17A1. *Biochem. Biophys. Res. Commun.* **443,** 179–184
25. Gregory, M., Mak, P. J., Sligar, S. G., and Kincaid, J. R. (2013) Differential hydrogen bonding in human CYP17 dictates hydroxylation versus lyase chemistry. *Angew. Chem. Int. Ed. Engl.* **52,** 5342–5345
26. Miller, S. L., Wright, J. N., Corina, D. L., and Akhtar, M. (1991) Mechanistic studies on pregnene side-chain cleavage enzyme (17α-hydroxylase-17,20-lyase) using ^{18}O. *J. Chem. Soc. Chem. Commun.* **1991,** 157–159
27. Akhtar, M., Corina, D. L., Miller, S. L., Shyadehi, A. Z., and Wright, J. N. (1994) Incorporation of label from $^{18}O_2$ into acetate during side-chain cleavage catalysed by cytochrome $P450_{17\alpha}$ (17α-hydroxylase-17,20-lyase). *J. Chem. Soc., Perkin Trans.* **1,** 263–267
28. Lee-Robichaud, P., Shyadehi, A. Z., Wright, J. N., Akhtar, M. E., and Akhtar, M. (1995) Mechanistic kinship between hydroxylation and desaturation reactions: acyl-carbon bond cleavage promoted by pig and human CYP17 ($P\text{-}450_{17\alpha}$; 17α-hydroxylase-17,20-lyase). *Biochemistry* **34,** 14104–14113
29. Akhtar, M., Lee-Robichaud, P., Akhtar, M. E., and Wright, J. N. (1997) The impact of aromatase mechanism on other P450s. *J. Steroid Biochem. Mol. Biol.* **61,** 127–132
30. Akhtar, M., Wright, J. N., and Lee-Robichaud, P. (2011) A review of mechanistic studies on aromatase (CYP19) and 17α-hydroxylase-17,20-lyase (CYP17). *J. Steroid Biochem. Mol. Biol.* **125,** 2–12
31. Akhtar, M., and Wright, J. N. (2015) Acyl-carbon bond cleaving cytochrome P450 enzymes: CYP17A1, CYP19A1 and CYP51A1. *Adv. Exp. Med. Biol.* **851,** 107–130
32. Guengerich, F. P., Sohl, C. D., and Chowdhury, G. (2011) Multi-step oxidations catalyzed by cytochrome P450 enzymes: processive vs. distributive kinetics and the issue of carbonyl oxidation in chemical mechanisms. *Arch. Biochem. Biophys.* **507,** 126–134
33. Yoshimoto, F. K., and Guengerich, F. P. (2014) Mechanism of the third oxidative step in the conversion of androgens to estrogens by cytochrome P450 19A1 steroid aromatase. *J. Am. Chem. Soc.* **136,** 15016–15025
34. Caspi, E., Arunachalam, T., and Nelson, P. A. (1986) Biosynthesis of estrogens: Aromatization of (19*R*)-, (19*S*)-, and (19*S*)-[19-^{3}H,^{2}H,^{1}H]-3β-hydroxyandrost-5-en-17-ones by human placental aromatase. *J. Am. Chem. Soc.* **108,** 1847–1852
35. Swinney, D. C., and Mak, A. Y. (1994) Androgen formation by cytochrome P450 CYP17. Solvent isotope effect and pL studies suggest a role for protons in the regulation of oxene versus peroxide chemistry. *Biochemistry* **33,** 2185–2190
36. Cai, H., and Guengerich, F. P. (1999) Mechanism of aqueous decomposition of trichloroethylene oxide. *J. Am. Chem. Soc.* 1991, **121,** 11656–11663
37. Pallan, P. S., Nagy, L. D., Lei, L., Gonzalez, E., Kramlinger, V. M., Azumaya, C. M., Wawrzak, Z., Waterman, M. R., Guengerich, F. P., and Egli, M. (2015) Structural and kinetic basis of steroid 17α,20-lyase activity in teleost fish cytochrome P450 17A1 and its absence in cytochrome P450 17A2. *J. Biol. Chem.* **290,** 3248–3268
38. Chowdhury, G., Calcutt, M. W., Nagy, L. D., and Guengerich, F. P. (2012) Oxidation of methyl and ethyl nitrosamines by cytochrome P450 2E1 and 2B1. *Biochemistry* **51,** 9995–10007
39. Chowdhury, G., Calcutt, M. W., and Guengerich, F. P. (2010) Oxidation of *N*-nitrosoalkylamines by human cytochrome P450 2A6: sequential oxidation to aldehydes and carboxylic acids and analysis of reaction steps. *J. Biol. Chem.* **285,** 8031–8044
40. Auchus, R. J., and Miller, W. L. (1999) Molecular modeling of human P450c17 (17α-hydroxylase/17,20-lyase): insights into reaction mechanisms and effects of mutations. *Mol. Endocrinol.* **13,** 1169–1182
41. Lichtenberger, F., Nastainczyk, W., and Ullrich, V. (1976) Cytochrome P-450 as an oxene transferase. *Biochem. Biophys. Res. Commun.* **70,** 939–946
42. Kirk, D. N., Toms, H. C., Douglas, C., White, K. A., Smith, K. E., Latif, S., and Hubbard, R. W. (1990) A survey of the high-field ^{1}H NMR spectra of the steroid hormones, their hydroxylated derivatives, and related compounds. *J. Chem. Soc., Perkin Trans.* **2,** 1990, 1567–1594
43. Krauser, J. A., Voehler, M., Tseng, L. H., Schefer, A. B., Godejohann, M., and Guengerich, F. P. (2004) Testosterone 1β-hydroxylation by human cytochrome P450 3A4. *Eur. J. Biochem.* **271,** 3962–3969
44. Hayamizu, K., and Kamo, O. (1990) Complete assignments of the ^{1}H and ^{13}C NMR spectra of testosterone and 17 α-methyltestosterone and the ^{1}H parameters obtained from 600 MHz spectra. *Magn. Reson. Chem.* **28,** 250–256
45. Hamilton, N. M., Dawson, M., Fairweather, E. E., Hamilton, N. S., Hitchin, J. R., James, D. I., Jones, S. D., Jordan, A. M., Lyons, A. J., Small, H. F., Thomson, G. J., Waddell, I. D., and Ogilvie, D. J. (2012) Novel steroid inhibitors of glucose 6-phosphate dehydrogenase. *J. Med. Chem.* **55,** 4431–4445
46. Ortiz de Montellano, P. R. (1986) in *Cytochrome P-450* (Ortiz de Montellano, P. R., ed) pp. 217–271, Plenum Press, New York
47. Betancor, C., Francisco, C. G., Freire, R., and Suarez, E. (1988) The reaction of enols with superoxide anion radicals: preparation of tertiary [small α]-ketols. *J. Chem. Soc. Chem. Commun.* **1998,** 947–948
48. Pestovsky, O., and Bakac, A. (2004) Reactivity of aqueous Fe(IV) in hydride and hydrogen atom transfer reactions. *J. Am. Chem. Soc.* **126,** 13757–13764
49. Bakac, A. (2002) Reactions of superoxo and oxo metal complexes with aldehydes. Radical-specific pathways for cross-disproportionation of superoxometal ions and acylperoxyl radicals. *J. Am. Chem. Soc.* **124,** 9136–9144
50. Paraskevas, P. D., Sabbe, M. K., Reyniers, M. F., Papayannakos, N. G., and Marin, G. B. (2014) Kinetic modeling of α-hydrogen abstractions from unsaturated and saturated oxygenate compounds by hydrogen atoms. *J. Phys. Chem. A* **118,** 9296–9309
51. Basran, J., Efimov, I., Chauhan, N., Thackray, S. J., Krupa, J. L., Eaton, G., Griffith, G. A., Mowat, C. G., Handa, S., and Raven, E. L. (2011) The mechanism of formation of *N*-formylkynurenine by heme dioxygenases. *J. Am. Chem. Soc.* **133,** 16251–16257
52. Akhtar, M., Corina, D., Miller, S., Shyadehi, A. Z., and Wright, J. N. (1994)

Mechanism of the acyl-carbon cleavage and related reactions catalyzed by multifunctional P-450s: Studies on cytochrome P45017α. *Biochemistry* **33,** 4410–4418
53. Yoshimoto, F. K., Zhou, Y., Peng, H. M., Stidd, D., Yoshimoto, J. A., Sharma, K. K., Matthew, S., and Auchus, R. J. (2012) Minor activities and transition state properties of the human steroid hydroxylases cytochromes P450c17 and P450c21, from reactions observed with deuterium-labeled substrates. *Biochemistry* **51,** 7064–7077
54. Kadlubar, F. F., Morton, K. C., and Ziegler, D. M. (1973) Microsomal-catalyzed hydroperoxide-dependent C-oxidation of amines. *Biochem. Biophys. Res. Commun.* **54,** 1255–1261
55. Rittle, J., and Green, M. T. (2010) Cytochrome P450 compound I: capture, characterization, and C–H bond activation kinetics. *Science* **330,** 933–937
56. Mansuy, D., Bartoli, J. F., and Momenteau, M. (1982) Alkane hydroxylation catalyzed by metalloporphyrins: evidence for different active oxygen species with alkylhydroperoxides and iodosobenzene as oxidants. *Tetrahedron Lett.* **23,** 2781–2784
57. Lee, D. S., Yamada, A., Sugimoto, H., Matsunaga, I., Ogura, H., Ichihara, K., Adachi, S., Park, S. Y., and Shiro, Y. (2003) Substrate recognition and molecular mechanism of fatty acid hydroxylation by cytochrome P450 from *Bacillus subtilis*. Crystallographic, spectroscopic, and mutational studies. *J. Biol. Chem.* **278,** 9761–9767
58. Rude, M. A., Baron, T. S., Brubaker, S., Alibhai, M., Del Cardayre, S. B., and Schirmer, A. (2011) Terminal olefin (1-alkene) biosynthesis by a novel P450 fatty acid decarboxylase from *Jeotgalicoccus* species. *Appl. Environ. Microbiol.* **77,** 1718–1727
59. Fujishiro, T., Shoji, O., Nagano, S., Sugimoto, H., Shiro, Y., and Watanabe, Y. (2011) Crystal structure of H_2O_2-dependent cytochrome $P450_{SP\alpha}$ with its bound fatty acid substrate: Insight into the regioselective hydroxylation of fatty acids at the α position. *J. Biol. Chem.* **286,** 29941–29950
60. Joo, H., Lin, Z., and Arnold, F. H. (1999) Laboratory evolution of peroxide-mediated cytochrome P450 hydroxylation. *Nature* **399,** 670–673
61. Renneberg, R., Scheller, F., Ruckpaul, K., Pirrwitz, J., and Mohr, P. (1978) NADPH and H_2O_2-dependent reactions of cytochrome P-450LM compared with peroxidase catalysis. *FEBS Lett.* **96,** 349–353
62. Renneberg, R., Damerau, W., Jung, C., Ebert, B., and Scheller, F. (1983) Study of H_2O_2-supported *N*-demethylations catalyzed by cytochrome P-450 and horseradish peroxidase. *Biochem. Biophys. Res. Commun.* **113,** 332–339
63. Nordblom, G. D., and Coon, M. J. (1977) Hydrogen peroxide formation and stoichiometry of hydroxylation reactions catalyzed by highly purified liver microsomal cytochrome P-450. *Arch. Biochem. Biophys.* **180,** 343–347
64. Renneberg, R., Capdevila, J., Chacos, N., Estabrook, R. W., and Prough, R. A. (1981) Hydrogen peroxide-supported oxidation of benzo[*a*]pyrene by rat liver microsomal fractions. *Biochem. Pharmacol.* **30,** 843–848
65. Holm, K. A., Engell, R. J., and Kupfer, D. (1985) Regioselectivity of hydroxylation of prostaglandins by liver microsomes supported by NADPH versus H_2O_2 in methylcholanthrene-treated and control rats: formation of novel prostaglandin metabolites. *Arch. Biochem. Biophys.* **237,** 477–489
66. Guengerich, F. P., Vaz, A. D., Raner, G. N., Pernecky, S. J., and Coon, M. J. (1997) Evidence for a role of a perferryl-oxygen complex, FeO^{3+}, in the *N*-oxygenation of amines by cytochrome P450 enzymes. *Mol. Pharmacol.* **51,** 147–151
67. Auchus, R. J., Lee, T. C., and Miller, W. L. (1998) Cytochrome b_5 augments the 17,20-lyase activity of human P450c17 without direct electron transfer. *J. Biol. Chem.* **273,** 3158–3165
68. Lacroix, D., Sonnier, M., Moncion, A., Cheron, G., and Cresteil, T. (1997) Expression of CYP3A in the human liver: evidence that the shift between CYP3A7 and CYP3A4 occurs immediately after birth. *Eur. J. Biochem.* **247,** 625–634
69. Niwa, T., Yabusaki, Y., Honma, K., Matsuo, N., Tatsuta, K., Ishibashi, F., and Katagiri, M. (1998) Contribution of human hepatic cytochrome P450 isoforms to regioselective hydroxylation of steroid hormones. *Xenobiotica* **28,** 539–547
70. Kirschner, M. A., Wiqvist, N., and Diczfalusy, E. (1966) Studies on oestriol synthesis from dehydroepiandrosterone sulphate in human pregnancy. *Acta Endocrinol.* **53,** 584–597
71. Stevenson, D. E., Wright, J. N., and Akhtar, M. (1988) Mechanistic consideration of P-450 dependent enzymic reactions: Studies on oestriol biosynthesis. *J. Chem. Soc. Perkin Trans.* **1,** 2043–2052
72. Cheng, Q., Sohl, C. D., Yoshimoto, F. K., and Guengerich, F. P. (2012) Oxidation of dihydrotestosterone by human cytochromes P450 19A1 and 3A4. *J. Biol. Chem.* **287,** 29554–29567
73. Petrunak, E. M., DeVore, N. M., Porubsky, P. R., and Scott, E. E. (2014) Structures of human steroidogenic cytochrome P450 17A1 with substrates. *J. Biol. Chem.* **289,** 32952–32964
74. Yoshimoto, F. K., Peng, H. M., Zhang, H., Anderson, S. M., and Auchus, R. J. (2014) Epoxidation activities of human cytochromes P450c17 and P450c21. *Biochemistry* **53,** 7531–7540
75. Walsh, C. (1979) *Enzymatic Reaction Mechanisms,* p. 121, W. H. Freeman & Co., San Francisco
76. DeVore, N. M., and Scott, E. E. (2012) Structures of cytochrome P450 17A1 with prostate cancer drugs abiraterone and TOK-001. *Nature* **482,** 116–119
77. Yosca, T. H., Rittle, J., Krest, C. M., Onderko, E. L., Silakov, A., Calixto, J. C., Behan, R. K., and Green, M. T. (2013) Iron(IV) hydroxide pK_a and the role of thiolate ligation in C–H bond activation by cytochrome P450. *Science* **342,** 825–829
78. Davydov, R., Strushkevich, N., Smil, D., Yantsevich, A., Gilep, A., Usanov, S., and Hoffman, B. M. (2015) Evidence that compound I is the active species in both the hydroxylase and lyase steps by which P450scc converts cholesterol to pregnenolone: EPR/ENDOR/cryoreduction/annealing studies. *Biochemistry* **54,** 7089–7097
79. Gottlieb, H. E., Kotlyar, V., and Nudelman, A. (1997) NMR chemical shifts of common laboratory solvents as trace impurities. *J. Org. Chem.* **62,** 7512–7515
80. Gillam, E. M., Baba, T., Kim, B. R., Ohmori, S., and Guengerich, F. P. (1993) Expression of modified human cytochrome P450 3A4 in *Escherichia coli* and purification and reconstitution of the enzyme. *Arch. Biochem. Biophys.* **305,** 123–131
81. Hosea, N. A., Miller, G. P., and Guengerich, F. P. (2000) Elucidation of distinct ligand binding sites for cytochrome P450 3A4. *Biochemistry* **39,** 5929–5939
82. Hanna, I. H., Teiber, J. F., Kokones, K. L., and Hollenberg, P. F. (1998) Role of the alanine at position 363 of cytochrome P450 2B2 in influencing the NADPH- and hydroperoxide-supported activities. *Arch. Biochem. Biophys.* **350,** 324–332
83. Guengerich, F. P. (2005) Reduction of cytochrome b_5 by NADPH-cytochrome P450 reductase. *Arch. Biochem. Biophys.* **440,** 204–211
84. Pallan, P. S., Wang, C., Lei, L., Yoshimoto, F. K., Auchus, R. J., Waterman, M. R., Guengerich, F. P., and Egli, M. (2015) Human cytochrome P450 21A2, the major steroid 21-hydroxylase: Structure of the enzyme·progesterone substrate complex and rate-limiting C–H bond cleavage. *J. Biol. Chem.* **290,** 13128–13143
85. Pechurskaya, T. A., Lukashevich, O. P., Gilep, A. A., and Usanov, S. A. (2008) Engineering, expression, and purification of "soluble" human cytochrome P45017α and its functional characterization. *Biokhimiia* **73,** 806–811
86. Sandhu, P., Baba, T., and Guengerich, F. P. (1993) Expression of modified cytochrome P450 2C10 (2C9) in *Escherichia coli*, purification, and reconstitution of catalytic activity. *Arch. Biochem. Biophys.* **306,** 443–450
87. Vijaykumar, D., Mao, W., Kirschbaum, K. S., and Katzenellenbogen, J. A. (2002) An efficient route for the preparation of a 21-fluoro progestin-16α,17α-dioxolane, a high-affinity ligand for PET imaging of the progesterone receptor. *J. Org. Chem.* **67,** 4904–4910
88. Saltzman, H., and Sharefkin, J. G. (1973) Organic Synthesis, Collective Volume 5. Iodosobenzene. *Org. Syn.* **1963,** 43, 60
89. Foust, G. P., Burleigh, B. D., Jr., Mayhew, S. G., Williams, C. H., Jr., and Massey, V. (1969) An anaerobic titration assembly for spectrophotometric use. *Anal. Biochem.* **27,** 530–535
90. Guengerich, F. P., Krauser, J. A., and Johnson, W. W. (2004) Rate-limiting steps in oxidations catalyzed by rabbit cytochrome P450 1A2. *Biochemistry* **43,** 10775–10788
91. Guengerich, F. P. (2014) in *Hayes' Principles and Methods of Toxicology* (Hayes, A. W., and Kruger, C. L., eds) 6th Ed., pp. 1905–1964, CRC Press-Taylor & Francis, Boca Raton, FL
92. Macdonald, T. L., Gutheim, W. G., Martin, R. B., and Guengerich, F. P. (1989) Oxidation of substituted *N*,*N*-dimethylanilines by cytochrome P-450: Estimation of the effective oxidation-reduction potential of cytochrome P-450. *Biochemistry* **28,** 2071–2077
93. Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology,* pp. 253 and 276, McGraw-Hill, New York