Unusually Facile Aromatization of 2β-Hydroxy-19-oxo-4-androstene-3,17-dione to Estrone. Implications in Estrogen Biosynthesis

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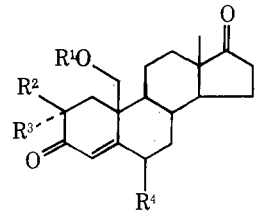
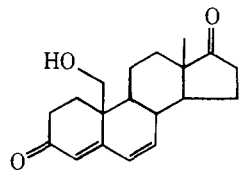
Abstract: 2β-Hydroxy-19-oxo-4-androstene-3,17-dione, synthesized as a potential intermediate in estrogen biosynthesis, undergoes a unique rapid and complete conversion to estrone in the presence of water at neutral or basic pH. The mechanism for this aromatization is postulated and its possible participation in the biological androgen to estrogen conversion is discussed.

The biosynthesis of the female sex hormone proceeds *via* the transformation of the C-19 neutral steroids to the C-18 phenolic estrogens. The aromatization reaction involves expulsion of the angular C-19 methyl group as formic acid[1] and the stereospecific loss of the 1β[2] and 2β hydrogens.[3] A complex of microsomal enzymes referred to as the aromatase is responsible for these transformations, and oxygen and NADPH are required as cofactors in the ratio of 3 mol each for each 1 mol of estrogen formed.[4] This stoichiometry is consistent with the participation of three enzymic hydroxylations in the overall transformation. Two of these have now been identified to take place on the C-19 methyl group as the initial steps in the aromatization process leading successively to the 19-hydroxy and 19-aldehyde intermediates.[5] The location and nature of the third hydroxylation are presently unknown, but its logical involvement in the C-1, C-2 hydrogen elimination suggested C-2 as its possible site. Earlier attempts to utilize 2β-hydroxy-4-androstene-3,17-dione as an estrogen precursor failed,[6] but this appeared to us to be inconclusive since the 2-hydroxylated compound may not be a suitable substrate for the C-19 hydroxylations which are the initial and requisite steps in estrogen biosynthesis. To test the hypothesis of C-2 hydroxylation participation in the aromatization process, therefore, substrates containing oxygen functions at *both* C-2 and C-19 were required. This paper describes the synthesis of the epimeric 2-hydroxy derivatives of androst-4-en-3-one containing also a hydroxy or an aldehyde function at C-19. The uniquely facile aromatization of one of these compounds to estrone is explored in detail, and its possible role and significance in the biosynthesis of estrogens are discussed.

There are two general routes available for the introduction of a C-2 acetoxy group into a Δ^4-3-keto steroid.[7] One is the reaction of a 6-bromo derivative with potassium acetate in glacial acetic acid, and the other is direct acetoxylation with lead tetraacetate. The former method was selected in this instance since it appeared to offer greater access to the less stable 2β-acetoxy isomer. Reaction of 19-acetoxy-4-androstene-3,17-dione (**1b**) with *N*-bromosuccinimide in carbon tetrachloride led to the corresponding 6β-bromo derivative **2**. The location of the bromine in **2** was confirmed *via* dehydrobromination with δ-collidine and subsequent hydrolysis which provided the known 19-hydroxy-4,6-androstadiene-3,17-dione (**3**).[8] The β-orientation of the bromine at C-6 was evident from the nmr spectral data.[9] When the bromo compound **2** was refluxed briefly in glacial acetic acid in the presence of potassium acetate, two isomeric products could be obtained after separation. The major product was assigned the structure 2β,19-diacetoxy-4-androstene-3,17-dione (**5b**), and the minor one was therefore the corresponding 2α-acetoxy derivative **6b**. The structure



1a, $R^1 = R^2 = R^3 = R^4 = H$
b, $R^1 = Ac$; $R^2 = R^3 = R^4 = H$
2, $R^1 = Ac$; $R^2 = R^3 = H$; $R^4 = Br$
5a, $R^1 = R^3 = R^4 = H$; $R^2 = OH$
b, $R^1 = Ac$; $R^2 = OAc$; $R^3 = R^4 = H$
c, $R^1 = R^3 = R^4 = H$; $R^2 = OSi(CH_3)_2$-*t*-Bu
6a, $R^1 = R^2 = R^4 = H$; $R^3 = OH$
b, $R^1 = Ac$; $R^2 = R^4 = H$; $R^3 = OAc$



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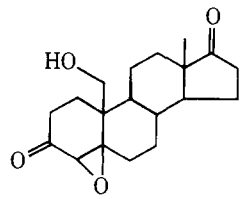

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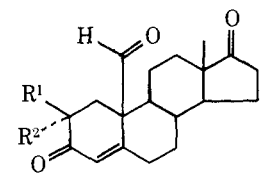
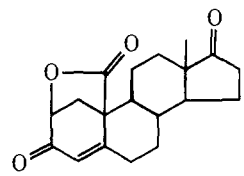
assignments of the two products and in particular the orientation of the acetoxy group at C-2 followed from their nmr and circular dichroism spectra. Crystallographic evidence has been presented that ring A of 2β-hydroxytestosterone is conformationally distorted into an inverted half-chair,[10] presumably because of the 1,3-diaxial interaction of the 2β-hydroxy and 19-methyl groups. This conformational distortion produces major changes in the CD and ORD spectra and also more subtle nmr distinctions.[11] The CD spectrum of the 2β isomer **5b** showed a positive Cotton effect in the n → π* region ($[\theta]_{324}$ +4800) and a negative one in the π → π* region ($[\theta]_{242}$ −49,100), comparable to the ORD data reported for 2β-hydroxytestosterone. The CD spectrum of the 2α epimer **6b**, on the other hand, was qualitatively similar to that of the starting material **1a** and showed a negative Cotton effect in the n → π* region ($[\theta]_{327}$ −6100) and a positive one in the π → π* region ($[\theta]_{234}$ +42,200). The signal for the C-2 hydrogen in **5b** in the nmr appeared at 5.28 ppm as a quartet (J = 8.4, 10.8 Hz) while that in **6b** appeared at 5.73 ppm also as a quartet (J = 6.0, 13.2 Hz). The similar coupling constants observed in the 2α and 2β hydrogens in **5b** and **6b**, respectively, suggest similar axial configurations for each, which is consistent with a conformational inversion of ring A in the 2β-acetoxy derivative **5b**.

Brief alkaline hydrolysis of the 2β-acetate in **5b** yielded 2β,19-dihydroxy-4-androstene-3,17-dione (**5a**), one of the desired compounds. The hydrolysis proceeded without isomerization or rearrangement since reacetylation regenerated the 2β-acetoxy derivative **5b**.

The synthetic route starting with the 6-bromo compound **2** was not suitable for the preparation of larger quantities of the 2α-hydroxy derivative and therefore an alternative route based on an abnormal acid-catalyzed reaction of 4β,5β-epoxy-3-keto steroids[12] was attempted. Sulfuric acid rearrangement of 4β,5β-epoxy-19-hydroxyandrostane-3,17-dione (**4**),[13] which in turn was obtained from alkaline hydrogen peroxide oxidation of 19-hydroxy-4-androstene-3,17-dione (**1a**), gave 2α,19-dihydroxy-4-androstene-3,17-dione (**6a**) in moderate yield. Acetylation of **6a** provided the diacetate **6b** identical in all respects with that obtained as the minor product from the 6-bromo pathway, which further served to confirm the structure assignments of **5b** and **6b**.

With the epimeric 2,19-diols of **5a** and **6a** at hand, the corresponding 19-aldehydes were still required. Direct oxidation of the 2α,19-diol **6a** with the chromium trioxide-pyridine complex proceeded smoothly and selectively to give the 2α-hydroxy-19-oxo-4-androstene-3,17-dione (**7**), which





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7, $R^1 = H$; $R^2 = OH$
a, $R^1 = OH$; $R^2 = H$
b, $R^1 = OSi(CH_3)_2$-*t*-Bu; $R^2 = H$

exhibited the formyl proton singlet resonance at 9.95 ppm. Similar oxidation of the 2β-hydroxy derivative **5a**, however, failed to stop at the aldehyde stage and yielded only the 2β,19-lactone **8**, which exhibited uv maxima at 230 and 264 nm, indicative of spatial interaction of the lactone and α,β-conjugated keto systems.[14] In the nmr spectrum of **8**, the 2α hydrogen appeared as a broad doublet at 4.65 ppm ($J_{1\beta,2\alpha}$ = 5Hz, $J_{1\alpha,2\alpha}$ = 3 Hz) reflecting its equatorial configuration and the conformational change of ring A in this compound due to the constraints of the lactone bond. Despite numerous other attempts with several selective oxidants, the 19-aldehyde could not be obtained directly from the 2β,19-diol. Participation of the 2β-hydroxyl may have been responsible for this failure, and selective blocking of the 2β-hydroxyl was therefore required to permit transformation of the 19-hydroxyl group to the aldehyde. Treatment of **5a** with a limited quantity of dimethyl-*tert*-butylsilyl chloride in dimethylformamide with imidazole as the catalyst[15] proceeded selectively to give the 2β-dimethyl-*tert*-butylsilyl monoether **5c** in good yield. Oxidation of the ether **5c** with the chromium trioxide-pyridine reagent did indeed yield the 19-aldehyde **9b**, as shown by the presence of the formyl proton singlet at 9.83 ppm in the nmr spectrum. Removal of the blocking silyl group was accomplished in a mixture of acetic acid, water, and tetrahydrofuran, and the desired 2β-hydroxy-19-oxo-4-androstene-3,17-dione (**9a**) was obtained. Resilylation of **9a** produced the silyl ether **9b**, confirming that no rearrangement or isomerization had occurred during the removal of the silyl group. The nmr spectrum of **9a** exhibited the formyl proton singlet at 9.61 ppm, and the 2α proton resonance appeared as a quartet at 4.20 ppm (J = 6, 8 Hz), indicative of its pseudoaxial configuration and hence of the continued conformational inversion of ring A in this compound.

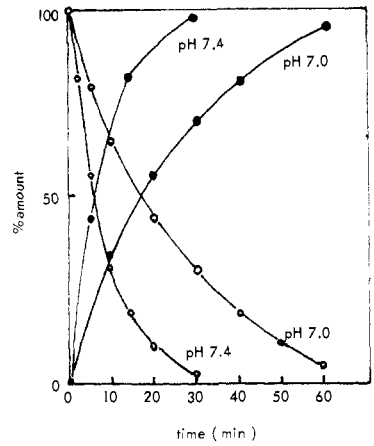

Figure 1. The disappearance of 2β-hydroxy-19-oxo-4-androstene-3,17-dione (**9a**) (- O -) and the appearance of estrone (- ● -) at 23°C monitored at 250 and 280 nm, respectively. The initial concentration of **9a** was 7×10^{-5} *M* in 0.05 *M* phosphate buffer containing 18% ethanol.

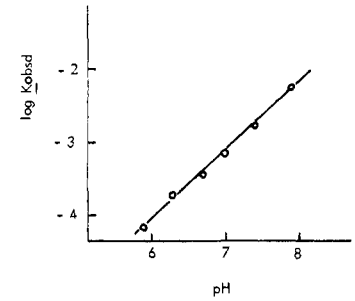

Figure 2. Rates of aromatization at 23° as a function of pH.

It was noted that in the process of removing the silyl ether group from **9b** to generate the 2β-hydroxy-19-aldehyde **9a** a quantity of estrone was also formed. This evidence of aromatization under unusually mild conditions prompted us to investigate further the behavior of the four 2,19-dioxy derivatives **5a**, **6a**, **7** and **9a** under various other conditions. Preliminary experiments revealed that all four compounds were stable at room temperature in organic solvents such as ethanol and chloroform. In the presence of water at neutral or alkaline pH, the two epimeric 2,19-diols **5a** and **6a** as well as the 2α-hydroxy-19-aldehyde **7** were not aromatized, but the 2β-hydroxy-19-aldehyde **9a** was rapidly and essentially completely converted to estrone. The nature and yield of the aromatization product were most conveniently obtained in a microscale reaction by means of radioisotope dilution procedures described in the Experimental Section. General base catalysis of the reaction was suggested by a similar conversion of **9a** to estrone in water and in several other buffer systems. The kinetics of the aromatization at 23° at various pH's were measured in 0.05 *M* phosphate buffer containing 18% ethanol. The rate of the reaction could be followed by the decrease of uv absorption of the α,β-unsaturated ketone in **9a** at 250 nm and the increase of the phenolic maximum at 280 nm.

Changes at these wavelengths with time at pH 7 and 7.4 are typical of those observed and are illustrated in Figure 1. The reaction is apparently first order, and the calculated rate constants are listed in Table I, and are plotted as a function of pH in Figure 2. In a single experiment the half-time of the aromatization at pH 7 at 37° was 90 sec.

The facile aromatization of the 2β-hydroxy-19-aldehyde **9a** and the lack of aromatization of the epimeric 2α-hydroxy-19-aldehyde **7** and of the 2,19-dihydroxy derivatives

Table I. Rate Constants of Estrone from **9a** at 23°

pH	$10^3 K_{obsd}$, 10^{-3} sec^{-1}	pH	$10^3 K_{obsd}$, 10^{-3} sec^{-1}
5.9	0.08	7.0	0.69
6.3	0.19	7.4	1.8
6.7	0.38	7.9	5.5

5a and **6a** imply a unique mechanism for the reaction involving participation of the 2β-hydroxy and 19-aldehyde functions. A proposed scheme for the conversion of **9a** to estrone is shown in Figure 3. The process is initiated by attack of the hydroxyl anion on the C-19 aldehyde followed by an anchimerically assisted elimination[16] of the 2β-hydroxy group. The dienone intermediate then collapses to estrone[17] by previously defined pathways. The anchimeric assistance is only possible because of the conformational inversion of ring A in the 2β-substituted compounds which brings the 1β hydrogen into proximity to the oxygen of the C-19 aldehyde. Such a relationship does not exist in the 2α-hydroxy-19-aldehyde structure which has a normal ring A conformation, and the equatorial orientation of the 1β hydrogen removes it from the proximity of the C-19 aldehyde, and therefore this compound does not aromatize. The 2β,19-diol **5a**, on the other hand, lacks the 19-aldehyde function where the process is initiated. Support for the proposed mechanism is currently being sought by defining experimentally the stereochemistry of the C-1 hydrogen elimination during the aromatization process.

The uniquely facile chemical aromatization of a compound whose synthesis was carried out because it was considered a probable intermediate in estrogen biosynthesis raises an obvious question about the possible role of this nonenzymatic aromatization in estrogen biosynthesis. Experimental testing of the estrogen precursor role of 2β-hydroxy-19-aldehyde **9a** is complicated by its very rapid and massive nonenzymatic aromatization to estrone under either physiological *in vivo* conditions or those of *in vitro* incubations with aromatase preparations, and it has therefore as yet not been possible to prove that the 2β-hydroxy-19-aldehyde structure actually participates in the biological process. Further biological studies are now under way to elucidate this point, but at the present time there is suggestive albeit circumstantial evidence, detailed below, that 2β-hydroxylation of a Δ^4-3-keto-19-aldehyde represents the last enzymatic reaction in the estrogen biosynthetic sequence with the nonenzymatic aromatization of the 2β-hydroxy-19-aldehyde intermediate being the ultimate step in estrogen formation. The 2β-hydroxy-19-aldehyde **9a** is the only compound that undergoes rapid aromatization at physiological conditions, and it is also the only one whose structure conforms to the known facts of estrogen biosynthesis. It is in accord with the stoichiometry and the sequence of three enzymatic hydroxylations involved in estrogen biosynthesis with the 2β hydroxylation succeeding the two prior ones responsible for generating the C-19 aldehyde. It is also in agreement with β stereochemistry of C-2 hydrogen elimination during biological aromatization[18] since the enzymatic 2β-hydroxylation would proceed *via* replacement of the 2β hydrogen. The rational mechanism proposed for the aromatization of the 2β-hydroxy-19-aldehyde structure specifically requires the elimination of the 1β hydrogen, a stereochemistry which is also in accord with the biological facts. Finally, the chemical aromatization of **9a** declines rapidly at a pH below 7, becoming negligible at pH 5. Similarly, the biosynthesis of estrogens with aromatase preparation is maximal at pH 7–7.4 and declines precipitously at lower pH values.

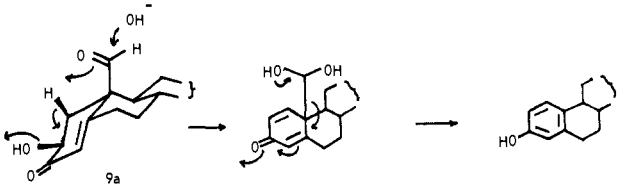

Figure 3. Postulated mechanism for base-catalyzed aromatization of 2β-hydroxy-19-oxo-4-androstene-3,17-dione.

The concurrences noted above may be coincidental, but they offer support for the concept of an intermediate role of the 2β-hydroxy-19-aldehyde in estrogen biosynthesis and provide an impetus for the ongoing biological studies designed to resolve the question.

Experimental Section

Melting points were obtained on a micro hot-stage apparatus and are uncorrected. Optical rotations were measured in $CHCl_3$. Infrared spectra were measured on a Beckman IR-9 infrared spectrophotometer as KBr pellets. Nuclear magnetic resonance spectra were obtained in $CDCl_3$ with TMS as an internal standard, using a Varian EM-360 nmr spectrometer. Circular dichroism spectra were recorded on a Cary 60 spectrophotometer in methanol. Ultraviolet spectra measurements were carried out on a Cary 15 spectrophotometer in ethanol.

19-Acetoxy-6β-bromo-4-androstene-3,17-dione (2). To a solution of 800 mg of 19-acetoxy-4-androstene-3,17-dione (**1b**) in 25 ml of carbon tetrachloride was added 700 mg of *N*-bromosuccinimide, and the mixture was refluxed in the absence of light for 1.5 hr. The reaction was cooled, and the precipitate was filtered off. After evaporation of solvent, crystallization from ether gave 320 mg of **2**: mp 138–139°; $[\alpha]^{24}D$ +95.2° (*c* 0.42); ir 1745, 1670, 1620, 1245 cm^{-1}; nmr δ 0.99 (3 H, s, 18-CH_3), 2.00 (3 H, s, 19, $OCOCH_3$), 4.40 (1 H, d, *J* = 11.5 Hz, one of 19-CH_2OAc), 4.60 (1 H, q, *J* = 1.0, 11.5 Hz, one of 19-CH_2OAc), 5.02 (1 H, q, *J* = 2.0, 4.0 Hz, 6α-H), 6.06 (1 H, s, 4-H). *Anal.* Calcd for $C_{21}H_{27}O_4Br$: C, 59.58; H, 6.43. Found: C, 59.75; H, 6.52.

Dehydrobromination of 2 with γ-Collidine. A solution of **2** (25 mg) in 1 ml of γ-collidine was refluxed for 1 hr. The reaction was diluted with ether, washed with 5% HCl, 5% $NaHCO_3$, and H_2O, and dried over anhydrous Na_2SO_4. The oily product was hydrolyzed with 1 *N* methanolic KOH under nitrogen for 20 min. After the usual work-up, the product was purified by preparative tlc (cyclohexane–ethyl acetate) and recrystallized from CH_2Cl_2–hexane to give 5 mg of 19-hydroxy-4,6-androstadiene-3,17-dione (**3**), mp 198–200°. The infrared spectrum was identical with that of an authentic sample.

2β,19-Diacetoxy-4-androstene-3,17-dione (5b) and 2α,19-Diacetoxy-4-androstene-3,17-dione (6b). To a solution of 480 mg of compound **2** in 8 ml of glacial AcOH was added 1.2 g of potassium acetate, and the mixture was refluxed for 12 min. The reaction mixture was then poured into ice–water, extracted with ether, washed with cold 10% K_2CO_3 and H_2O, and dried over anhydrous Na_2SO_4. After evaporation of the solvent, recrystallization from ether gave 35 mg of **5b**: mp 202–203°; $[\alpha]^{22}D$ +62.0° (*c* 0.40); uv 239 nm (ε 15,700); ir 1745, 1700, 1630, 1240, 1210 cm^{-1}; nmr δ 0.90 (3 H, s, 18-CH_3), 2.00 (3 H, s, 19-$OCOCH_3$), 2.13 (3 H, s, 2β-$OCOCH_3$), 3.96 and 4.50 (1 H, d, *J* = 11.5 Hz, 19-CH_2OAc), 5.28 (1 H, q, *J* = 8.4, 10.8 Hz, 2α-H), 5.90 (1 H, s, 4-H). *Anal.* Calcd for $C_{23}H_{30}O_6$: C, 68.68; H, 7.51. Found: C, 68.54; H, 7.51.

The residue from the mother liquors was recrystallized from ether and gave 8 mg of **6b**: mp 141–142°; $[\alpha]^{23}D$ +178.3° (*c* 0.42); uv 237 nm (ε 15,800); ir 1745, 1695, 1629, 1225 cm^{-1}; nmr δ 0.92 (3 H, s, 18-CH_3), 2.08 (3 H, s, 19-$OCOCH_3$), 2.16 (3 H, s, 2β-$OCOCH_3$), 4.25 and 4.73 (1 H, d, *J* = 11.5 Hz, 19-CH_2OAc), 5.73 (1 H, q, *J* = 13.2, 6.0 Hz, 2β-H), 5.92 (1 H, s, 4-H). *Anal.* Calcd for $C_{23}H_{30}O_6$: C, 68.68; H, 7.51. Found: C, 68.63; H, 7.44.

4β,5β-Epoxy-19-hydroxyandrostane-3,17-dione (4). To a solution of 220 mg of 19-hydroxy-4-androstene-3,17-dione (**1a**) in 2 ml of methanol were added 0.4 ml of 10% NaOH and 0.2 ml of 30% H_2O_2, and the solution was allowed to stand at 0° for 1 hr. The reaction mixture was diluted with ethyl acetate, washed with H_2O,

and dried over anhydrous Na_2SO_4. After work-up, the product was recrystallized from CH_2Cl_2–hexane to give 200 mg of **4**: mp 201–203°; $[\alpha]^{24}D$ +231.9° (*c* 0.40); nmr δ 0.90 (3 H, s, 18-CH_3), 2.90 (1 H, s, 4α-H), 3.78 and 4.12 (1 H, d, J = 11.5 Hz, 19-CH_2OH). *Anal.* Calcd for $C_{19}H_{26}O_4$: C, 71.67; H, 8.23. Found: C, 71.80; H, 8.27.

2β,19-Dihydroxy-4-androstene-3,17-dione (5a). To a solution of 10 mg of **5b** in 0.8 ml of methanol was added 0.06 ml of 1 *N* KOH, and the solution was allowed to stand at room temperature for 10 min under nitrogen. The reaction mixture was neutralized with 1 *N* AcOH and extracted with ethyl acetate. After purification by preparative tlc (benzene–ethyl acetate), recrystallization from ether–hexane gave 4.5 mg of **5a**: mp 140–142°; $[\alpha]^{24}D$ +64.7° (*c* 0.30); uv 242 nm (ϵ 11,250); ir 3430, 1730, 1665, 1620 cm^{-1}; nmr δ 0.90 (3 H, s, 18-CH_3), 3.60 (1 H, d, J = 11.5 Hz, one of 19-CH_2OH), 4.06 (2 H, 2α-H and one of 19-CH_2OH), 5.93 (1 H, s, 4-H). *Anal.* Calcd for $C_{19}H_{26}O_4$: C, 71.67; H, 8.23. Found: C, 71.84; H, 8.58. Reacetylation of **5a** gave the diacetate identical by infrared with **5b.**

2α,19-Dihydroxy-4-androstene-3,17-dione (6a). A solution of **4** (200 mg) in 10 ml of acetone containing 0.2 ml of concentrated H_2SO_4 and 0.6 ml of H_2O was allowed to stand at room temperature for 66 hr. The reaction mixture was diluted with ethyl acetate, washed with cold 5% $NaHCO_3$ and H_2O, and dried over anhydrous Na_2SO_4. After purification by preparative tlc (benzene–ethyl acetate), recrystallization from acetone gave 40 mg of **6a**: mp 201–203°; $[\alpha]^{23}D$ +177.1° (*c* 0.35); uv 241 nm (ϵ 12,400); ir 3480, 1740, 1670, 1620 cm^{-1}; nmr δ 0.90 (3 H, s, 18-CH_3), 4.02 (2 H, s, 19-CH_2OH), 4.65 (1 H, q, J = 6.0, 13.2 Hz, 2β-H), 5.93 (1 H, s, 4-H). *Anal.* Calcd for $C_{19}H_{26}O_4$: C, 71.67; H, 8.23. Found: C, 71.52; H, 8.04. Acetylation of **6a** gave the diacetate **6b**, identical in all respects with that obtained from **2.**

2α-Hydroxy-19-oxo-4-androstene-3,17-dione (7). A solution of **6a** (25 mg) in 0.2 ml of pyridine and 0.5 ml of a 10% CrO_3–pyridine complex was allowed to stand at room temperature for 20 min. The reaction mixture was diluted with ethyl acetate, washed with cold 10% AcOH, 5% $NaHCO_3$, and H_2O, and dried over anhydrous Na_2SO_4. After purification by preparative tlc (cyclohexane–ethyl acetate), recrystallization from CH_2Cl_2–hexane gave 11 mg of **7**: mp 205–207°; $[\alpha]^{20}D$ +241.5° (*c* 0.21); ir 3490, 1738, 1710, 1680, 1610 cm^{-1}; uv 245 nm (ϵ 10,000); nmr δ 0.90 (3 H, s, 18-CH_3), 4.15 (1 H, q, J = 6, 13 Hz, 2β-H), 6.03 (1 H, s, 4-H), 9.95 (1 H, s, 19-CHO). *Anal.* Calcd for $C_{19}H_{24}O_4 \cdot \frac{1}{2}H_2O$: C, 70.13; H, 7.74. Found: C, 70.73; H, 7.69.

Oxidation of 5a to 2β-Hydroxy-4-androstene-3,17-dion-19-oic Acid 2,19-Lactone (8). A solution of **5a** (20 mg) in 0.2 ml of pyridine and 0.5 ml of a 10% CrO_3–pyridine complex was allowed to stand at room temperature for 20 min. After work-up in the same manner as described above, recrystallization from CH_2Cl_2–hexane gave 8 mg of **8**: mp 191–193°; $[\alpha]^{20}D$ +404.8° (*c* 0.21); ir 1780, 1740, 1700, 1603 cm^{-1}; uv 230 (ϵ 6600), 264 nm (ϵ 6800); nmr δ 1.06 (3 H, s, 18-CH_3), 4.65 (1 H, broad d, J = 5 Hz, 2α-H), 5.83 (1 H, s, 4-H). *Anal.* Calcd for $C_{19}H_{22}O_4$: C, 72.59; H, 7.05. Found: C, 71.99; H, 7.10.

2β,19-Dihydroxy-4-androstene-3,17-dione 2-(Dimethyl-*tert*-butylsilyl Ether) (5c). To a solution of 140 mg of **5a** in 4.5 ml of DMF were added 200 mg of dimethyl-*tert*-butylsilyl chloride and 330 mg of imidazole, and the solution was allowed to stand at room temperature for 1 hr. The reaction mixture was diluted with ether, washed with H_2O, and dried over anhydrous Na_2SO_4. After purification by preparative tlc (benzene–ether), recrystallization from ether–hexane gave 79 mg of **5c**: mp 132–134°; $[\alpha]^{23}D$ +89.2° (*c* 0.33); ir 3420, 1741, 1698, 1628, 1138, 870, 840, 785 cm^{-1}; uv 242 nm (ϵ 14,700); nmr δ 0.13 and 0.20 (3 H, s, $SiCH_3$), 0.90 (12 H, s, 18-CH_3 and *tert*-butyl), 3.5–4.2 (3 H, 2α-H and 19-CH_2OH), 5.94 (1 H, s, 4-H). *Anal.* Calcd for $C_{25}H_{40}O_4Si$: C, 69.40; H, 9.32. Found: C, 69.49; H, 9.36.

2β-Hydroxy-19-oxo-4-androstene-3,17-dione Dimethyl-*tert*-butylsilyl Ether (9b). A solution of **5c** (72 mg) in 1 ml of pyridine and 2 ml of a 10% CrO_3–pyridine complex was allowed to stand at room temperature for 30 min. After the usual work-up and purification by preparative tlc (cyclohexane–ethyl acetate), recrystallization from ether–hexane gave 51 mg of **9b**: mp 187–188°; $[\alpha]^{23}D$ +171.0° (*c* 0.35); ir 1740, 1720, 1680, 1620, 838, 782 cm^{-1}; uv 250 nm (ϵ 10,700); nmr δ 0.01 and 0.10 (3 H, s, Si-CH_3), 0.82 (9 H, s, *tert*-butyl), 0.85 (3 H, s, 18-CH_3), 4.02 (1 H, broad s, 2α-4), 5.92 (1 H, s, 4-H), 9.83 (1 H, s, 19-CHO). *Anal.* Calcd for $C_{25}H_{38}O_4Si$: C, 69.72; H, 8.89. Found: C, 70.08; H, 8.82.

2β-Hydroxy-19-oxo-4-androstene-3,17-dione (9a). A solution of **9b** (128 mg) in 16 ml of a AcOH–H_2O–THF mixture (3:1:0.5) was allowed to stand at 60° for 26 hr. The reaction mixture was diluted with ethyl acetate, washed with H_2O, and dried over anhydrous Na_2SO_4. After purification by preparative tlc (cyclohexane–ethyl acetate), recrystallization gave 20 mg of **9a**: mp 166–168°; $[\alpha]^{25}D$ +135.3° (*c* 0.09); ir 3280, 1745, 1687, 1133 cm^{-1}; uv 245 nm (ϵ 11,000); nmr δ 0.97 (3 H, s, 18-CH_3), 4.20 (1 H, q, J = 6, 8 Hz, 2α-H), 6.00 (1 H, s, 4-H), 9.61 (1 H, s, 19-CHO). *Anal.* Calcd for $C_{19}H_{24}O_4$: C, 72.12; H, 7.65. Found: C, 71.76; H, 7.74.

From less polar fraction, a mixture of 20 mg of recovered **9b** and 10 mg of estrone was obtained. Estrone was isolated and identified as its dimethyl-*tert*-butylsilyl ether. Resilylation of **9a** with dimethyl-*tert*-butylsilyl chloride in DMF containing imidazole gave a silyl ether identical in the infrared with **9b.**

Analysis of Nature and Yield of Aromatization Product of 9a by Radioisotope Dilution Procedures. A solution of 240 μg of **9a** in 0.1 ml of methanol and 10 ml of 0.05 *M* phosphate buffer (pH 7.0) was allowed to stand at room temperature for 50 min. A solution of [4-^{14}C]estrone (23,000 cpm; 0.1 μg) and 1 g of sodium borohydride in 10 ml of methanol was then added, and the mixture was allowed to stand at 0° for 2 hr. After work-up and tlc separation, the estradiol region, visualized by uv absorption, was eluted and acetylated with 0.1 ml of acetic-^3H anhydride (specific activity: 138×10^3 cpm/μ equiv) in 0.2 ml of pyridine. After evaporation of the excess reagent, estradiol diacetate (20 mg) was added, and the mixture was first purified by preparative tlc and then recrystallized to a constant isotope ratio of ^3H to ^{14}C (8.2). The amount of estrone produced from **9a** was calculated to be 184 μg (90%) according to the equation

$$\text{amount of estrone } (\mu g) = [^3H]/[^{14}C]\,aM/yn$$

where a = cpm of added estrone-^{14}C; M = molecular weight of estrone; y = specific activity of acetic-^3H anhydride (cpm/μequiv); n = number of acetate groups in derivative, which in the case of estradiol diacetate is 2.

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Note Added in Proof. A portion of this work has appeared in a preliminary form.[19]

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The Catalytic Mechanism of the Manganese-Containing Superoxide Dismutase of *Escherichia coli* Studied by Pulse Radiolysis[1a]

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Abstract: The dismutation of O_2^-, catalyzed by *Escherichia coli* Mn dismutase, has been investgated. O_2^- was generated in formate aqueous solutions by pulse radiolysis. When the initial concentration of O_2^-, $[O_2^-]_0$, is less than 10 times the total concentration of the dismutase, $[E]_0$, a reaction first order in both $[O_2^-]$ and $[E]_0$ is observed, the apparent reaction rate constant of which is $1.5 \pm 0.15 \times 10^9\ M^{-1}\ sec^{-1}$. When $[O_2^-]_0/[E]_0 > 15$, a biphasic process is observed. Under these conditions only about 15 O_2^- radical ions per each dismutase molecule react with a relatively fast rate. Excess O_2^- is removed by a less efficient reaction, also first order in $[E]_0$ and nearly first order in $[O_2^-]$, which has an apparent rate constant $1.6 \pm 0.25 \times 10^8\ M^{-1}\ sec^{-1}$. The results are interpreted in terms of four oxidation and reduction reactions, such as: (i) $E + O_2^- \rightarrow E^- + O_2$, $k = 1.3 \pm 0.15 \times 10^9\ M^{-1}\ sec^{-1}$; or (i)′ $E + O_2^- + 2H^+ \rightarrow E^+ + H_2O_2$; (ii) $E^- + O_2^- + 2H^+ \rightarrow E + H_2O_2$, $k = 1.6 \pm 0.6 \times 10^9\ M^{-1}\ sec^{-1}$; or (ii)′ $E^+ + O_2^- \rightarrow E + O_2$; (iii) $E^- + O_2^- \rightarrow E^{2-} + O_2$, $k \simeq 2 \times 10^8\ M^{-1}\ sec^{-1}$; or (iii)′ $E + O_2^- \rightarrow E^- + O_2$; (iv) $E^{2-} + O_2^- + 2H^+ \rightarrow E^- + H_2O_2$, $k \simeq 1 \times 10^7\ M^{-1}\ sec^{-1}$; or (iv)′ $E^- + O_2^- + 2H^+ \rightarrow E + H_2O_2$. E^+, E, E^-, and E^{2-} may represent forms of enzymes in which Mn^{IV}, Mn^{III}, Mn^{II}, and Mn^{I} are respectively present. Alternative mechanisms involving additional oxidation states of the Mn are discussed. After the end of the catalytic process the high enzyme activity is fully regenerated within less than 30 sec.

Enzymatic catalysis of superoxide dismutation has been evident since the work of McCord and Fridovich.[2] The rate of catalysis and its mechanism have been extensively investigated for bovine superoxide dismutase.[3-6] It has been shown that alternate reduction and oxidation of the copper atoms is involved.[6]

Recently, competition experiments have shown a high catalytic efficiency of *Escherichia coli* mangano superoxide dismutase.[7] In this manuscript, we report a direct observation of the catalysis, followed with the aid of the pulse radiolysis technique.

When an oxygenated aqueous solution containing formate ions is irradiated the following reactions take place.

$$H_2O \longrightarrow e_{aq}^-, H, OH, H_3O^+, OH^-, H_2O_2, H_2 \quad (1)$$

$$e_{aq}^- + O_2 \longrightarrow O_2^- \quad k_2 = 2 \times 10^{10}\ M^{-1}\ sec^{-1}\ [8] \quad (2)$$

$$H + O_2 \longrightarrow HO_2 \quad k_3 = 2 \times 10^{10} M^{-1}\ sec^{-1}\ [9] \quad (3)$$

$$H + HCO_2^- \longrightarrow H_2 + CO_2^- \quad k_4 = 5 \times 10^8 M^{-1}\ sec^{-1}\ [10] \quad (4)$$

$$OH + HCO_2^- \longrightarrow H_2O + CO_2^- \quad k_5 = 3 \times 10^9 M^{-1}\ sec^{-1}\ [11] \quad (5)$$

$$CO_2^- + O_2 \longrightarrow CO_2 + O_2^-\ [t2] \quad (6)$$

$$HO_2 \rightleftharpoons H^+ + O_2^- \quad K = 1.6 \times 10^{-5} M\ [13, 14] \quad (7)$$

As a result, at sufficiently high [formate], O_2^- radical ions (in equilibrium with HO_2) are produced as the only radical species within less than 1 μsec. O_2^- radical ions decay away to form O_2 and H_2O_2. Under our conditions, reaction 8 competed efficiently with both (9) and (10). In the following we report the enhanced decay of the O_2^- radical ions, as followed in the uv, upon the addition of the *E. coli* Mn dismutase.

$$HO_2 + O_2^- \xrightarrow{H^+} H_2O_2 + O_2 \quad k_8 = 8.5 \times 10^7 M^{-1}\ sec^{-1}\ [t3, t4] \quad (8)$$

$$HO_2 + HO_2 \longrightarrow H_2O_2 + O_2 \quad k_9 = 6.7 \times 10^5 M^{-1}\ sec^{-1}\ [13, 14] \quad (9)$$

$$O_2^- + O_2^- \xrightarrow{2H^+} H_2O_2 + O_2 \quad k_{10} \cong 10^2 M^{-1}\ sec^{-1}\ [14] \quad (10)$$

Experimental Section

The pulse radiolysis apparatus and the optical detection system have been described previously.[14] The solutions contained 10^{-4} *M* ethylenediaminetetraacetate (EDTA) and 10^{-2} *M* formate unless otherwise stated. Blank experiments were always carried out before the injection of the enzyme from a stock solution. In the blank solutions, O_2^- decayed away by two parallel reactions. One of these was a second-order process and the other an apparent first-order process. Similar observations were reported and discussed previously.[13] These results indicate a fairly low level of impurities in our solutions. Phosphate buffer was used to adjust the pH. *E. coli* dismutase has been isolated and purified as before.[15] Other materials were of high purity grade and were used as received. All solutions were saturated with O_2 (Matheson). Unless stated otherwise, measurements were carried out at 290 nm. Scattered light measurements[6] were carried out for each of the enzyme concentrations used. The scattered light was less than 10% and was ignored.