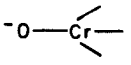
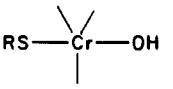
D2 pathway is considered unlikely.

The rates of reaction of chromate and hydrogen chromate with thioglycolic acid were anomalously high when compared to the other thiols examined. One possible explanation for the higher rate observed with thioglycolic acid is a chelation effect. Involvement of both the carboxyylate and thiol groups in the formation of the chromium(VI) thio ester would be favored for thioglycolic acid which could form a five-membered chelate ring with chromium. In contrast, chelation of chromium would not be favored with cysteine, *N*-acetylcysteine, and 3-mercaptopropionic acid which would form six-membered rings or homocysteine which would form a seven-membered ring. Another possible explanation is that with thioglycolic acid, the internal geometry of the transition state is the most favorable for proton transfer from the bound carboxylic acid, –COOH, to –CrOH.

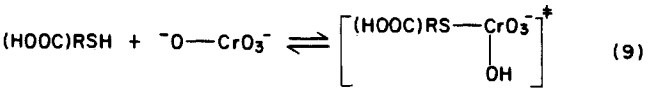
In conclusion, these studies have shown that the ligand substitution reactions of chromium(VI) with thiols which result in the formation of chromium(VI) thio esters involves attack by the protonated thiol on either chromate (eq 9) or hydrogen chromate with proton transfer as the rate-determining step. Protonation of chromate inhibits attack by RSH on



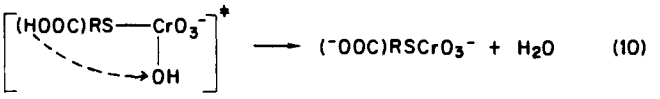
and formation of the five-coordinate transition state,



Loss of water and formation of the chromium(VI) thio ester at high pH (>3) is facilitated by proton transfer from a bound carboxylic acid group to –CrOH (eq 10). Under acidic conditions H_3O^+ facilitates water elimination. In the absence of a proton donor (H_3O^+, –COOH, $-NH_3^+$), the reaction is very slow since hydroxide is the leaving group. The following mechanism is proposed for these reactions:



(9)



(10)

Acknowledgment. This work was supported by the donors of the Petroleum Research Fund, administered by the American Chemical Society, by Grant NP-519 from the American Cancer Society, by PHS Grant CA34869 awarded by the National Cancer Institute, DHHS, and by an A. P. Sloan Research Fellowship. The helpful advice of Walter H. Stockmayer is gratefully acknowledged. The assistance of Thomas H. Hampton in all aspects of computerized data collection and analysis is gratefully acknowledged.

Registry No. Cr(VI), 18540-29-9; glutathione, 70-18-8; L-cysteine ethyl ester, 3411-58-3; L-cysteine, 52-90-4; cysteamine, 60-23-1; coenzyme M, 45127-11-5; homocysteine, 6027-13-0; *N*-acetyl-L-cysteine, 616-91-1; coenzyme A, 85-61-0; 2-mercaptoethanol, 60-24-2; thioglycolate, 68-11-1.

(30) Albert, A. *Biochem. J.* **1952**, *50*, 690–698.
(31) Reuben, D. M. E.; Bruice, T. C. *J. Am. Chem. Soc.* **1976**, *98*, 114–121.
(32) *Merck Index*, 10th ed.; Windholz, M., Ed.; Merck: Rahway, NJ; 1983; p 685.
(33) "Lange's Handbook of Chemistry", 12th ed.; Dean, J. A., Ed.; McGraw-Hill: New York, 1979; pp 5–17–5–41.
(34) Brown, H. C.; McDaniel, D. H.; Häfliger, O. In *Determination of Organic Structures by Physical Methods*; Braude, E. A., Nachod, F. C., Eds.; Academic: New York, 1955; Vol 1, pp 567–662.

Biosynthesis of Estrogens: Aromatization of (19*R*)-, (19*S*)-, and (19*RS*)-[19-^{3}H,^{2}H,^{1}H]-3β-Hydroxyandrost-5-en-17-ones by Human Placental Aromatase†

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Contribution from The Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545. *Received June 19, 1985*

Abstract: It is shown, with the use of (19*R*)- and (19*S*)-[19-^{3}H,^{2}H,^{1}H]-3β-hydroxyandrost-5-en-17-ones, that the "first" C-19 hydroxylation by human placental aromatase involves a normal kinetic isotope effect $k_H > k_D > k_T$. The hydroxylation proceeds stereospecifically in the retention mode; i.e., the incoming hydroxyl assumes the orientation of the displaced (isotopic) hydrogen atom.

In the 1940s and 1950s, it was recognized that cholesterol[1] and particularly testosterone and other C_{19} steroids[2] can be metabolized to estrogens. However, the mechanism of the removal of the C-19 methyl group and the mode of aromatization of ring A were not understood. It was Meyer[3] who demonstrated that the first step of the aromatization process involved C-19 hydroxylation of an androgen substrate. Subsequently, it was shown that the extrusion of the 10β-methyl involves three oxidative steps, each of which requires 1 mol of O_2 and 1 mol of NADPH.[4] Whether the aromatization proceeds in discrete stages or is a continuous process occurring on the enzyme surface is not clear and is being debated.[5]

†Work supported by NIH Grant HD 14906. A preliminary account of this work was published (*J. Am. Chem. Soc.* **1983**, *104*, 6987).
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(1) (a) Werbin, H.; Plotz, J.; LeRoy, G. V.; Davis, E. M. *J. Am. Chem. Soc.* **1957**, *79*, 1012. (b) Savard, K.; Andrec, K.; Brooksbank, B. W. L.; Reyneri, C.; Dorfman, R. I. *J. Biol. Chem.* **1958**, *231*, 765.
(2) (a) Nathanson, I. T.; Towne, L. E. *Endocrinology* **1939**, *25*, 754. (b) Bagget, B.; Engel, L. L.; Savard, K.; Dorfman, R. I. *J. Biol. Chem.* **1956**, *221*, 931.
(3) Meyer, A. S. *Experientia* **1955**, *11*, 99; *Biochim. Biophys. Acta* **1955**, *17*, 441.
(4) Thompson, E. A., Jr.; Siiteri, P. K. *J. Biol. Chem.* **1974**, *249*, 5364.
(5) Kelly, W. G.; Judd, D.; Stolee, A. *Biochemistry*, **1977**, *16*, 140.

0002-7863/86/1508-1847$01.50/0

Table I

mixture of 19-chiral and [4-^{14}C]-3β-hydroxyandrost-5-en-17-one		% conversion to estradiol		recovered, $\times 10^{-3}$ dpm		TCOOH/ THO	steric mode of 19-hydroxylation
		[19-T,D,H] androgen	[4-^{14}C]-androgen	TCOOH	THO		
19R[a]	1	4.86	5.69	133	92	1.45	retention
(F = 64)	2	4.75	6.42	130	89	1.47	retention
	3	4.70	5.93	127	90	1.41	retention
19RS[b]	1	4.83	5.70	101	108	0.94	
(F = 50)	2	5.02	6.46	100	103	0.97	
	3	4.95	6.34	99	108	0.92	
19S[c]	1	4.59	5.82	87	128	0.68	retention
(F = 33)	2	4.66	5.40	88	126	0.70	retention
	3	4.70	5.64	88	128	0.69	retention

[a] Corrected by using control I. [b] Corrected by using control II. [c] Corrected by using conditions of control I; average recovery of H^{14}COOH, 93.7%, and ^{3}HOH, 79.7%.

However, convincing evidence was obtained that the 19-alcohol is converted to a 19-aldehyde[6] which, in turn, is aromatized. The conversion of the 19-alcohol to an aldehyde is not an "alcohol dehydrogenase"–NADP (or NAD) mediated reaction and requires a mole each of O_2 and NADPH.[4,6] It was therefore postulated that the alcohol is converted to a "19-diol" which is then dehydrated to yield the 19-aldehyde.[6] Evidence was obtained showing that the second C-19 hydroxylation proceeds *without an isotope effect*[7] and involves the stereospecific *abstraction of the 19 pro-R hydrogen*[8-10] atom. Ultimately, the hydrogen atoms abstracted in the "first" and "second" hydroxylation are exchanged with the medium.[6] In addition, it was demonstrated that the *oxygen atom* introduced in the "*second hydroxylation*" is eliminated in the dehydration leading to the aldehyde.[11] Finally, the sequence is completed by aromatization of the aldehyde, which requires 1 mol of O_2 and 1 mol of NADPH, and carbon-19 is extruded as formic acid. The resulting formic acid retains the 19 *pro-S* hydrogen of the parent 19-alcohol.[11]

Our objective was to determine the steric mode of the *initial* C-19 hydroxylation[3] by placental aromatase. Previously, we have proven that the steric mode of enzymatic hydroxylation at primary carbon atoms can be determined with the use of chiral methyl groups (labeled with ^{3}H, ^{2}H, and ^{1}H) provided that the oxygenation involves a kinetic (normal) hydrogen isotope effect ($k_H > k_D > k_T$) (^{2}H = D; ^{3}H = T).[12-14]

The deuterium[15] and tritium[16] kinetic isotope effects for the first C-19 hydroxylation of androgens by placental aromatase were investigated. The deuterium isotope effects[15] (K_H/K_D) for the hydroxylation of 19-CH_2D and 19-CD_3 are 2.3 and 3.2, respectively. The reported tritium isotope effect[16] (K_H/K_T) for hydroxylation of 19-CT_3 is 3.2, the same as that for 19-CD_3. The identical value of isotope effects for the hydroxylation of 19-CT_3 and 19-CD_3 is rather unusual since generally tritium isotope effects are significantly larger than the deuterium isotope effects.[17]

Results

The (19*R*)-, (19*S*)-, and (19*RS*)-[19-^{3}H,^{2}H,^{1}H]-3β-hydroxyandrost-5-en-17-ones required for these studies were synthesized by two routes.[18] The chiral compounds were converted to the corresponding androst-4-en-3,17-diones and treated with SeO_2–H_2O_2.[19,20] The derived seco-acid lactones were oxidized by the Kuhn-Roth method,[20] and the chirality of the recovered samples of acetic acid was determined by the malate–synthetase–fumarase procedure.[20,21,22] The acetic acids derived from the samples of (19*R*)-androgens (synthesized by two routes) showed[22] F = 63 and 65. The acetic acids obtained from the two samples of (19*S*)-androgens each showed F = 33. The 19*RS* samples showed F = 50 and 49. Assuming a linear correlation of the F value and diastereoisomeric purity, it can be calculated that of the chiral molecules present in the samples with F = 63 and 65, ca. 72.5% and ca. 76%, had 19R methyls, respectively. The Kuhn–Roth oxidation proceeded with the exchange of up to 5% of the protons of the produced acetic acid. It follows therefore that the chiral molecules present in the 19R samples had up to 78% and 81% molecules with 19R methyls, respectively. The two 19S specimens showed F = 33, indicating that ca. 79.5% of the chiral molecules had 19S methyls. This, when corrected for potential losses during Kuhn–Roth reaction, indicates that up to 85% of the chiral molecules had 19S methyls.

The enzymatic aromatization experiments were carried out in an identical manner in triplicate. Parallel with the incubations, control recovery experiments for each radioactive component (THO, TCOOH, [4-^{14}C]estradiol) were also carried out in triplicate. The procedure involved the incubation of a mixture of chiral [19-^{3}H,^{2}H,^{1}H]-3β-hydroxyandrost-5-en-17-one (2.1 μCi; 100 μg) and [4-^{14}C]-3β-hydroxyandrost-5-en-17-one (1 μCi; 35 μg) with human placental aromatase for 1 h in air at 35–37 °C. The reaction was terminated by acidification with phosphoric acid (0.74 M; 1 mL), frozen (liquid N_2) and lyophilized. The recovered mixture of tritiated water and formic acid was counted. The solution was then made alkaline (1.25 M NaOH) (phenolphthalein) frozen, and the tritiated water removed by lyophilization. The tritium content of the water and of the sodium formate was determined. The "nonvolatile" residue of the incubation was taken up in water, the steroids were extracted with ethyl acetate, and the phenolic fraction was isolated via partitioning with 1.25 M NaOH. The obtained estrogens were treated with $NaBH_4$, and the resulting mixture was fractionated by high-performance liquid chromatography. The estradiol fraction was collected and counted.

The results (Table I) are corrected for control recovery of the relevant radioactive component(s). The percent conversions of the 19-chiral substrates to estrogens are calculated from the *sum* of the (corrected) amounts of tritium in the formic acid and in water. For the three sets of experiments (Table I), the observed

(6) (a) Akhtar, M.; Skinner, S. J. M. *Biochem. J.* **1968**, *109*, 318. (b) Skinner, S. J. M.; Akhtar, M. *Biochem. J.* **1969**, *114*, 75.
(7) Miyaki, S.; Fishman, J. 7th International Congress of Endocrinology, July 1–7, 1984, Quebec City, Canada; Abstr. 1620.
(8) Arigoni, D.; Bataglia, R.; Akhtar, M.; Smith, T. *Chem. Commun.* **1975**, 185.
(9) Osawa, Y.; Shibata, K.; Rohrer, D.; Weeks, C.; Duax, W. L. *J. Am. Chem. Soc.* **1975**, *97*, 4400.
(10) Caspi, E.; Santaniello, E.; Patel, K.; Arunachalam, T.; Eck, C. R. *J. Am. Chem. Soc.* **1978**, *100*, 5223.
(11) Akhtar, M.; Calder, M. R.; Corina, D. L.; Wright, J. N. *Biochem. J.* **1982**, *201*, 569.
(12) Caspi, E.; Piper, J. U.; Shapiro, S. *Chem. Commun.* **1981**, 76; *Tetrahedron* **1981**, *37*, 3535.
(13) Caspi, E.; Shapiro, S.; Piper, J. U. *Chem. Commun.* **1981**, 1196. Shapiro, S.; Piper, J. U.; Caspi, E. *J. Am. Chem. Soc.* **1982**, *104*, 2301.
(14) Shapiro, S.; Arunachalam, T.; Caspi, E. *J. Am. Chem. Soc.* **1983**, *105*, 1642.
(15) Holland, H. L.; Taylor, G. J. *Can. J. Chem.* **1981**, *59*, 2809.
(16) Miyari, S.; Fishman, J. *Biochem. Biophys. Res. Commun.* **1983**, *117*, 392.
(17) Yakushin, F. S. *Russ. Chem. Rev. (Engl. Trans.)* **1962**, *31*, 123.
(18) Caspi, E.; Santaniello, E.; Patel, K.; Arunachalam, T. *Chem. Commun.* **1984**, 379.
(19) Caspi, E.; Balasubrahmanyan, S. N. *J. Org. Chem.* **1963**, *28*, 3383.
(20) Caspi, E.; Chang, C. C.; Bush, K. H.; Arunachalam, T. *Croat. Chimica Acta*, in press, special issue in honor of Prof. Mihailo Lj. Mihailovic.
(21) Cornforth, J. W.; Redmond, J. W.; Eggerer, H.; Buckel, W.; Gutchow, C. *Nature (London)* **1969**, *221*, 1212; *Eur. J. Biochem.* **1970**, *14*, 1. Luthy. J.; Retey, J.; Arigoni, D. *Nature (London)* **1969**, *221*, 1213.
(22) Floss, H. G.; Tsai, M. D. *Adv. Enzymol.* **1979**, *50*, 243.

ratio of the turnover of [4-^{14}C]androgen vs. the [19-C]TDH androgen is 1.24. This ratio, which should be an approximate measure of the overall isotope effect K_H/K_{TDH}, is considerably lower than expected. To assure ourselves of the functioning of the placental aromatase, we have measured the combined yields of the biosynthesized [^{14}C]estradiol and [^{14}C]estrone (as [^{14}C]-estradiol; see Experimental Section) with the omission of other biosynthesized [^{14}C]estrogens. This omission may be a reason for the observed low 1.24 ratio.

It is evident from the data in Table I that the results for each set of experiments are reproducible within a narrow range. In all instances, the aromatization of the internal reference [19-^{1}H$_3$;4-^{14}C]androgen was ca. 20% higher than that of the chiral [19-^{3}H,^{2}H,^{1}H]androgens (Table I, columns 2 and 3), which is consistent with the operation of an isotope effect. The average TCOOH/THO ratio for the 19RS substrate was 0.94, revealing *a nearly equal* distribution of the isotope in the two products (see below). The formic acid derived from the 19R substrate consistently and reproducibly contained more tritium than the water. The average TCOOH/HTO ratio derived from the 19R substrate is 1.44. In contrast, the THO derived from the aromatization of the 19S substrates contained more tritium than the formic acid, as evidenced by the average TCOOH/THO ratio of 0.69.

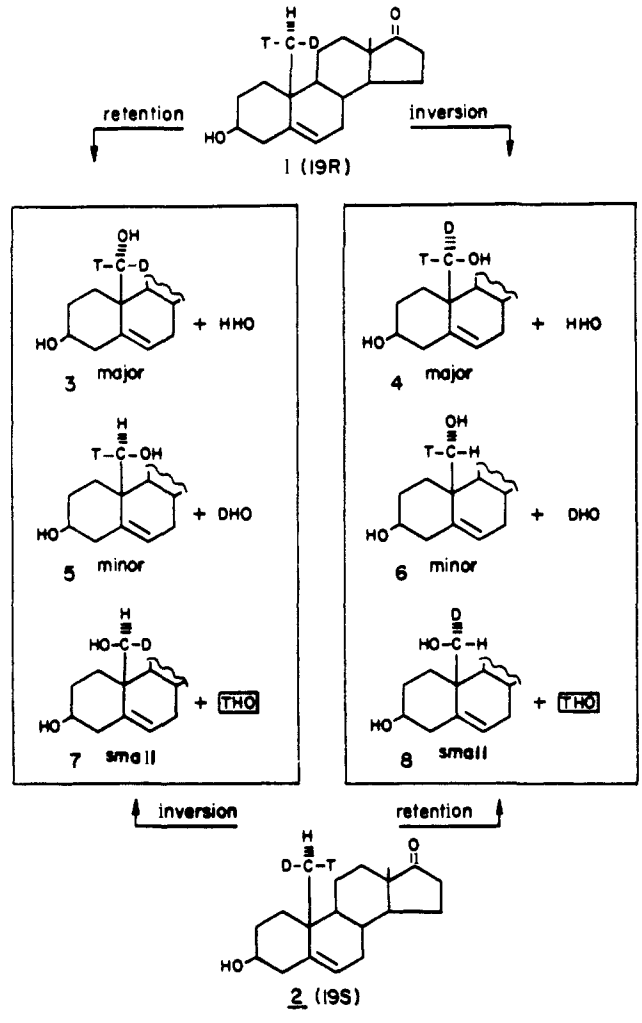
It may be noticed that 19R and 19S androgens had about the same optical purities and the results for these two substrates were nearly reciprocal. Reciprocal results could be expected if the once-initiated hydroxylation of the C-19 methyl of the substrate proceeds to completion of the aromatization or the "second" C-19 hydroxylation does not involve an isotope effect. The fact that the total amounts of tritium released as [TCOOH] + [THO] from the "same" amounts of 19-TDH substrates are about equal is in accord with the view that the *once-initiated* C-19-hydroxylation proceeds to completion of aromatization. In addition, the evidence that the second hydroxylation proceeds without an isotope effect[7] clearly establishes the fact that the "first" C-19 hydroxylation involves a normal kinetic isotope effect.

The results for the 19RS sample which indicated a nearly equal distribution of tritium in formic acid and water were of particular importance (see below), and we decided to reexamine the data for this substrate. Rather than to repeat the assays on the same material, a new sample of (19RS)-substrate was prepared as follows.[24] [19-^{3}H]-3β-Methoxy-10β-formylandrost-5-ene 17-ketal was reduced with an equimolar mixture of (*R*)- and (*S*)-Alpine boranes. The resulting 19RS alcohol was reacted with methyltriphenoxyphosphonium iodide and the obtained 19RS iodide was hydrogenolyzed with deuteriosuperhydride to give, after processing, the required (19*RS*)-(19-^{3}H,^{2}H,^{1}H]-3β-hydroxyandrost-5-en-17-one (F = 49). The 19RS substrate was incubated (in duplicate) with placental aromatase, and the tritiated formic acid and tritiated water were recovered and counted. The results were corrected for control recovery experiments which were carried out in parallel. The TCOOH/THO ratio was shown to be 0.91 and 0.85. It may be noticed that the average ratio for first determination 0.94 is in good agreement with the average results for the second experiment 0.88 (see below).

Discussion

The relation between tritium and deuterium isotope effects can be expressed[25] as $k_H/k_T = [k_H/k_D]^r$ where $1.58 \geq r \geq 1.33$. When k_H/k_D is small, the lower r value applies, while to temperature-independent isotope effects the higher r value applies.[26] In the "Swain equations" used for calculations of k_H/k_T, r = 1.442 is employed.[26] The Swain equation is applicable for calculation of tritium isotope effects of transformations at isotopically "similar" systems, e.g., $CH_2D \sim CH_2T$, $CD_3 \sim CT_3$ etc. Based on the results of the deuterium isotope effect[15] for the hydroxylation of 19-CD_3 k_H/k_D = 3.2, the calculated k_H/k_T for the hydroxylation of 19-CT_3 should be 5.35, rather than the reported[16] 3.2. This raises the question of the applicability of Swain's equation to the enzymatic C-19 hydroxylation or of the significance of the reported deuterium and/or tritium isotope effects.

Scheme I



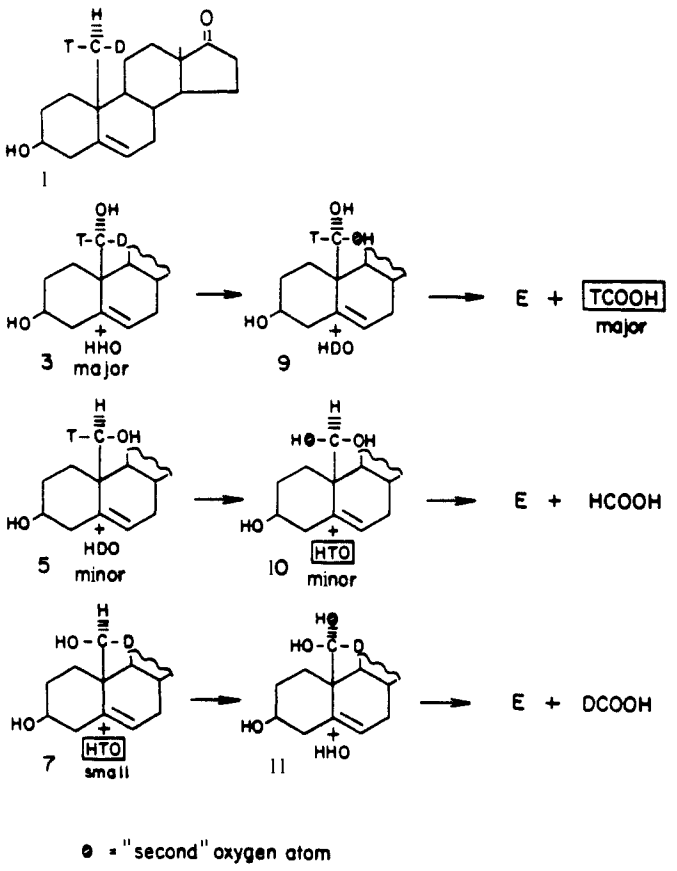
Theoretical Consideration of Oxidative Transformations at C-19. For the current studies, it was necessary to prove that *initial* C-19 hydroxylation involves kinetic isotope effects ($k_H > k_D > k_T$) and that these can be differentiated when the 19-methyl is chiral. We will review the results which could be expected for the various operational conditions of isotope effects. In analogy to previous observations,[12-14] we will first assume that the initial C-19 hydroxylation by placental aromatase involves isotope effects which can be differentiated. Our results (Table I) are consistent with this view.

The hydroxylation could proceed with retention, inversion, or racemization. Thus, "first" C-19 hydroxylation with retention of 19R-methyl (**1**) (Scheme I) will yield, following the abstraction of a hydrogen atom, the 19S 19-DT alcohol (Scheme I (**3**)) as the *major* product and, following the abstraction of a deuterium atom, the 19R 19-HT alcohol **5** as the *minor* product. Cleavage of a carbon–tritium bond will give the 19S 19-HD alcohol **7**. As indicted earlier, the displaced hydrogen atoms are exchanged with the protons of the water of the medium.[6] If the K_H/K_T is relatively *small*, significant amounts of 19-HD alcohol **7** will be formed and will result in the release of a corresponding amount of tritium in the water. In contrast, if K_H/K_T is *large*, an insignificant amount of **7** will be formed and the small amount of tritiated water produced at this stage could be disregarded.

Should the first hydroxylation of the 19R methyl (**1**) proceed with inversion, then the major product will be 19R 19-DT alcohol

(23) See, e.g.: Fishman, J.; Guzik, H.; Dixon, D. *Biochemistry* **1969**, *8*, 4304. Fishman, J.; Dixon, D. *Ibid.* **1967**, *6*, 1683.
(24) Arunachalam, T.; Santaniello, E.; Patel, K.; Caspi, E., unpublished results.
(25) Lewis, E. S.; Robinson, J. K. *J. Am. Chem. Soc.* **1968**, *90*, 4337.
(26) "The Chemists Companion. A Handbook of Practical Data, Techniques and References"; Wiley: New York, 1972; p 141.

Scheme II



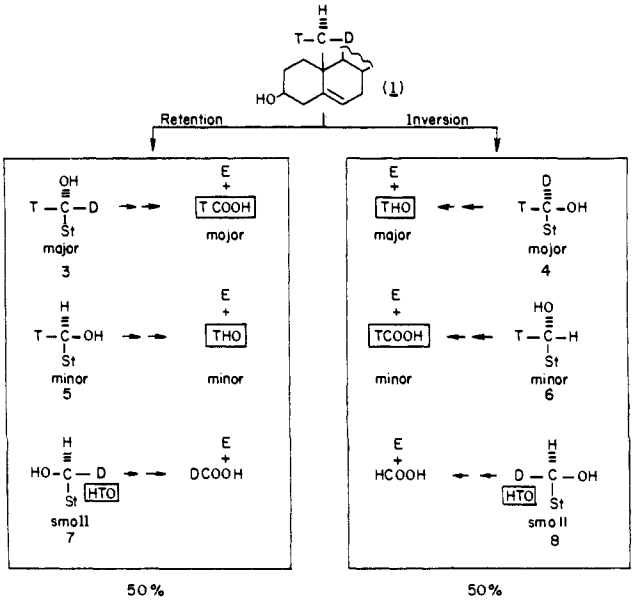
(Scheme I (4)) and the minor product will be the 19S 19-HT alcohol **6**. The yield of the 19R 19-HD alcohol **8** and of tritiated water produced at this stage will again depend on the magnitude of K_H/K_T.

The same reasoning is applicable to the hydroxylation of an androgen having a 19S methyl group (Scheme I (**2**)). In this instance, hydroxylation with inversion will produce alcohols **3**, **5** and **7**, which will correspond in composition and yield to alcohols obtained from hydroxylation of 19R methyl (**1**) with retention. In contrast, hydroxyation of the 19S methyl (**2**) with retention will give products **4**, **6**, and **8**, which correspond to products of hydroxylation of the 19R methyl (**1**) with inversion.

The next step of the aromatization process requires the introduction of a second hydroxyl at C-19. As indicated earlier, this apparently proceeds with complete utilization of the hydroxylated intermediate and *without* an isotope effect.[7] The second hydroxylation involves the stereospecific abstraction of the 19-*pro-R* hydrogen atoms of the 19-alcohols.[8-10] This will be exemplified for the case of 19-alcohols derived from the 19R methyl (**1**) by hydroxylation in the retention mode ($k_H > k_D > k_T$) (Scheme II). Thus, the second hydroxylation of 19S alcohol **3** will proceed with abstraction of deuterium to yield 19-tritiated diol **9** and HDO. Subsequent transformations of **9** will result in an estrogen and tritiated formic acid. Analogous second hydroxylation of the 19R alcohol **5** (Scheme II) will give **10**, and the eliminated tritium will be transferred to the water of the medium generating THO. Subsequently, the diol **10** will be aromatized to yield an estrogen and protiated formic acid. Since alcohol **7** is devoid of tritium, it need not be considered. If a *relatively large kinetic isotope effect* $k_H > k_D > k_T$ operates in the first hydroxylation, it is very likely that the formic acid will contain more tritium than the *total amount* of tritium in the water (see **7** and **10**; Scheme II). Should the *first* hydroxylation proceed in the same manner but with inversion (Scheme I (**4**–**8**)), *the water* will contain *more tritium* than the *formic acid*.

The same rationalization is applicable to the aromatization of the 19S substrate **2**. However, in this instance, the "first" hydroxylation in the retention mode will give alcohols **4**, **6**, and **8** (Scheme I) which, on aromatization, will yield *formic acid* which will contain *less tritium* than the *water*. In contrast, the first hydroxylation in the inversion mode will produce alcohols **3**, **5**, and **7**, which will aromatize to give *formic acid* that will contain *more tritium* than the *water*.

Scheme III



We will now consider the case of the "first" hydroxylation proceeding with an isotope effect and racemization (Scheme III). This implies that 50% of a chiral substrate, e.g., (19*R*)-**1**, will be hydroxylated with retention and 50% with inversion (Scheme III). Subsequently, the alcohols of each group will be aromatized to yield estrogens, TCOOH, and HTO. The isotope effect for the first hydroxylation with retention or inversion must be the same (Scheme III). In view of the fact that 19-hydroxylated intermediates are apparently completely aromatized and that the second C-19 hydroxylation proceeds without an isotope effect, the following distribution of tritium may be expected.

The amounts of tritium released in the aromatization of **3** and **4** (Scheme III) must be equal:

$$[\text{TCOOH}](3) = [\text{THO}](4)$$

Similarly, the amounts of tritium released in the aromatization of **5** and **6** must be equal:

$$[\text{TCOOH}](6) = [\text{THO}](5)$$

It follows, therefore, that the amounts of tritium in the formic acid derived from **3** and **6** are equal to the amount of tritium in the water derived from **4** and **5** (Scheme III):

$$[\text{TCOOH}](3 + 6) = [\text{THO}](4 + 5)$$

In other words,

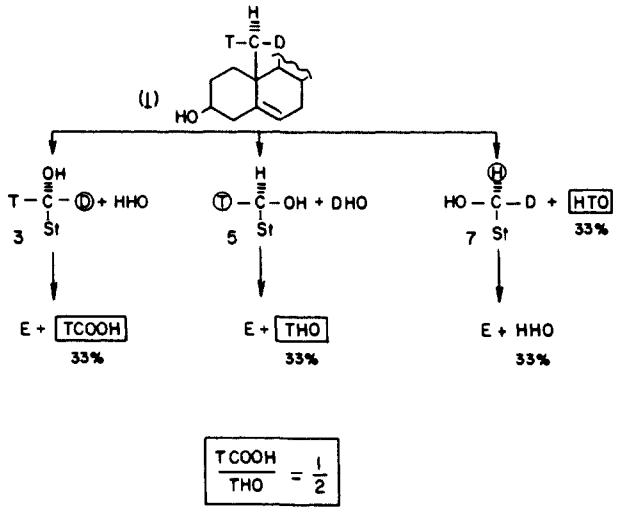
$$\frac{[\text{TCOOH}](3 + 6)}{[\text{THO}](4 + 5)} = 1$$

There remains still the factor of *tritiated water* generated in the formation of alcohols **7** and **8**. If the tritium isotope effect of the initial C-19 hydroxylation is small, then relatively large amounts of alcohols **7** and **8** will be obtained and significant amounts of tritiated water will be formed ([THO](**7** + **8**) large)). Consequently, the [TCOOH]/[THO] ratio will be significantly smaller than one.

$$\frac{[\text{TCOOH}](3 + 6)}{[\text{THO}](4 + 5) + [\text{THO}](7 + 8)} < 1$$

On the other hand, should the isotope effect of the initial hy-

Scheme IV



droxylation be large, only small amounts of tritiated water will be formed ([THO](7 + 8) small). Consequently, the ratio will be nearly one (TCOOH/THO ≤ 1), as we have observed (0.94 and 0.88).

The same reasoning is applicable to a 19S and a 19RS substrate (containing equal amounts of (19*R*)- and (19*S*)-androgens). In all instances the tritium will be distributed as discussed above.

In the event that the first C-19 hydroxylations will proceed without an isotope effect, the *three C-19* (isotopic) hydrogen atoms will have an *equal opportunity* to be displaced by a hydroxyl. The C-19 hydroxylation of, e.g., (19*R*)-**1** with retention, but without an isotope effect, is summarized in Scheme IV. Thus, 33% of the molecules will lose a hydrogen atom to yield **3** which, at the completion of the aromatization process, will give formic acid containing 33% tritium. The abstraction of deuterium (from 33% of the molecules) will give **5**, which will then aromatize and yield water containing 33% tritium. Similarly, displacement of tritium (from 33% of the molecules) will also give water containing 33% tritium. Consequently, at the termination of the aromatization process, 33% tritium will be located in the formic acid and 66% tritium will be found in the water (Scheme IV). Hence, in the absence of an isotope effect, irrespective of the chirality of the substrates 19R, 19S, or 19RS and irrespective of whether the hydroxylation proceeds with retention, inversion, or racemization, the ratio [TCOOH/THO] = $^1/_2$ will be constant.

It is apparent from the above discussion that the determination of the steric mode of the first C-19 hydroxylation depends critically on the determination of the ratio of tritium in formic acid and in water released from chiral 19-methyls in the aromatization process.

As mentioned earlier, for isotopically equivalently labeled molecules (CH_2D) ~ CH_2T, CD_3 ~ CT_3, etc.), hypothetically the tritium isotope effect can be calculated from the deuterium isotope effect with the use of Swain's equation. In the absence of data on isotope effects of chiral methyls [C-^{3}H,^{2}H,^{1}H], we will tentatively assume that the deuterium isotope effect of a [CH_1D_2]methyl can be used for the approximate calculation of the tritium isotope effect of a chiral methyl. Unfortunately, no data are available on the isotope effect of a [CH_1D_2]methyl. Consequently, for the current discussion, we will take the average (2.75) of the isotope effects[15] of [19-CH_2D]methyl (2.3) and [19-CD_3]methyl (3.2) as the approximate deuterium isotope effect of the [19-CHD_2]methyl. This value when applied to Swain's equation (r = 1.442) would give k_H/k_T = *4.30*. Thus, assuming the "equivalency" of [CHD_2] and [C-^{3}H,^{2}H,^{1}H], the calculated tritium isotope effect for the chiral 19-methyl would be 4.30. It follows, therefore, that the C-19 hydroxylation of **1** (Scheme I) in the retention mode will proceed with the abstraction of H/D/T at a ratio of 4.30/1.56/1. In other words, alcohols **3**, **5**, and **7** will be produced in 62.68%, 22.74%, and 14.58% yields, respectively. As indicated above, the second hydroxylation proceeds with complete utilization of the 19-hydroxylated intermediate and without an isotope effect.[7] The produced aldehyde is then rapidly aromatized.[11] Consequently, at the end of the process outlined in Scheme II, 62.68% tritium will be present in the formic acid and 37.32% (22.74 + 14.58) tritium will be in the water. In short, the calculated TCOOH/THO ratio for a 100% pure 19R sample is 1.68.

Table II. TCOOH/THO Ratios Calculated on the Basis of Swain's Equation, Using k_H/k_T = (2.75)1.442 = 4.3 (See Text for Details)

sample	F value	TCOOH/THO ratio calcd	TCOOH/THO ratio found (av)
19R	64	1.11	1.44
19RS	50	0.72	0.91
19S	33	0.44	0.69

Since our 19R sample showed F = 64, it may be inferred that it was a mixture of 75% (19*R*)-methylandrogens and 25% (19*S*)-methylandrogens.[22] The 19R and 19S specimens will be metabolized as described in Scheme I. Thus, 75% of the 19R compound is expected to give, at the termination of the aromatization, (62.68 × 0.75) = 47.01% TCOOH and (22.74 × 0.75) + (14.58 × 0.75) = 27.99% HTO. Similarly, 25% of the 19S component will give (22.74 × 0.25) = 5.69% TCOOH and (62.68 × 0.25) + (14.58 × 0.25) = 19.31% HTO. It follows that at the completion of the estrogen elaboration from the 19R substrate (F = 64), (47.01 + 5.69) = 52.7% tritium will be in TCOOH and (27.99 + 19.31) = 47.30% tritium will be in HTO. Hence, the TCOOH/THO ratio, calculated on the basis of Swain's equation, will be 52.7/47.30 = 1.11. The (19*S*)-androgen (F = 33) consists of 80% 19S and 20% 19R specimens. A similar calculation of the distribution of tritium indicates that 30.73% tritium will be in the TCOOH and 69.23% in HTO for a ratio TCOOH/HTO = 0.44. The calculated ratio for the 19RS substrate (F = 49) consisting of 50% 19R and 50% 19S specimens is TCOOH/THO = 0.75. The ratios of TCOOH/HTO, calculated on the basis of Swain's equation, are summarized in Table II. It is apparent that the calculated ratios (Table II) are systematically lower than the experimental results (Tables I and II).

Clearly, within a narrow range of experimental error, our results are reproducible. It is also evident that aromatization of the 19R substrate consistently gave formic acid which contained *more tritium than the water*. In contrast, the 19S substrate produced formic acid which *contained less tritium than the water*.

The TCOOH/THO ratios 0.94 and 0.88 (average 0.91) for the 19RS substrates are indicative of a nearly equal distribution (47.5% and 52.5%) of tritium in the formic acid and water. This is consistent with the view that a large isotope effect K_H/K_T operates in the first hydroxylation and therefore little tritium is released in this step.

The question arises how best to rationalize the observed, consistently higher [TCOOH]/[THO] ratios. It is likely that the value K_H/K_D = 2.75 is inappropriate for calculating the K_H/K_T for chiral methyls. Based on the calculated K_H/K_T, more tritiated water is presumably generated in the aromatization process (Scheme II), which results in a lowering of TCOOH/THO ratios. The other possibility, already mentioned in the previous paragraph, is that the K_H/K_T in the first C-19 hydroxylation is significantly higher and, therefore, only a small amount of alcohol (**7**) (Scheme I) is formed. Consequently, little tritiated water is produced at this stage of the reaction. Under these circumstances, completion of the aromatization process (Scheme II) will result in the release of tritiated formic acid from alcohol **3** and of tritiated water essentially only from alcohol **10**. Should this be the case, the TCOOH/THO ratio for the 19RS substrate will be nearly one, and the ratios for the 19R and 19S substrates will be reciprocal. These results are in accord with our experimental data.

In any event, our results unambiguously show that the first C-19 hydroxylation proceeds in the retention mode.

Experimental Section

Materials and Methods. Analytical grade (AR) reagents and solvents were used. Tritiated water, [^{14}C]formic acid, [1,2,6,7-^{3}H]estradiol, and

[4-^{14}C]-3β-hydroxyandrost-5-en-17-one were purchased from New England Nuclear, Inc., Boston, MA. Glucose-6-phosphate dehydrogenase, dithiothreitol, and NADP were obtained from Sigma Chemical Co., St. Louis, MO. Glucose-6-phosphate was supplied by Cal-biochem., San Diego, CA.

The radioactivity counting was done on a Packard TRI-CARB liquid scintillation spectrometer in either Liquifluor or Aquasol, both purchased from New England Nuclear, Boston, MA. High-performance liquid chromatography was carried out, using a Micromeritics Co. 7000 instrument equipped with a Model 750 solvent delivery system and Model 788 UV variable-wavelength detector. A silica-packed column (25 cm × 0.46 cm i.d.) (10–25-μm particles) purchased from Alltech Co., Deerfield, IL, was used. Ethanol (5%) in hexane or heptane was the mobile phase (1 mL/min), and the detector was set at 210 and 254 nm. For radioactivity scanning, the effluent from the UV detector was mixed continuously with Liquifluor (3 mL per 1 mL of effluent), and the mixture was then passed through a FLOW-ONE radioactive detector (Radiomatic Instrument, Inc., Tampa, FL) programmed for tritium and/or ^{14}C, as needed.

Enzyme Preparation. Full-term placentae were collected and placed on ice immediately after delivery and processed within *45 min*. Cotyledous tissue was weighed, minced, and homogenized in 50 mM phosphate buffer, pH 7.4, containing 0.25 M sucrose, using 1 mL of buffer for each 20 g of tissue. Debris and mitochondria were separated out by centrifugation at 15000*g* for 15 min. The supernate was decanted and centrifuged at 100000*g* for 70 min. Pellets were resuspended in buffer and recentrifuged at 100000*g* for 60 min and then resuspended in 50 mM Tris–maleic acid buffer, pH 7.4. The volume of Tris–maleic acid was chosen so that 1-mL aliquots contained the equivalent of 20 g of wet tissue. These 1-mL aliquots were then quick-frozen (liquid N_2) and stored at −20 °C for up to 3 months.[27]

The protein concentration was determined with the biuret assay,[28] using bovine serum albumin as a standard. Microsomal preparations contained between 40–50 mg of protein/20 g-equiv of wet tissue (i.e., 1 mL of final suspension).

Incubation. A mixture of [19-^{3}H,^{2}H,^{1}H]- (100 μg, 2.1 μCi) and [4-^{14}C]-3β-hydroxyandrost-5-en-17-ones (35 μg, 1.0 μCi) was dissolved in 4 mL of Tris–maleic acid buffer containing 2% ethanol, 3 mM NADPH, 15 mM glucose-6-phosphate, glucose-6-phosphate dehydrogenase, 1 μkat/mL (60 IU/mL), and 6 mM dithiothreitol. Reactions were initiated by the addition of 1 mL of the above mcirosomal suspension. Incubations were carried out in the air at 35–37 °C with mild shaking for 1 h and were terminated with 1 mL of 0.74 M phosphoric acid.

Control of Products Recovery. Two types of control recovery experiments were carried out in which [^{14}C]formic acid (0.025 μCi, 5 μg), THOH (0.025 μCi, 2.5 μg), and [2,4,6,7-^{3}H]estradiol (0.025 μCi, 3 μg) were included in addition to the reagents noted earlier. In the first set of controls, the mixtures were incubated (1 h; 37 °C) and the microsomal material was added just prior to the addition of the phosphoric acid. The second set of controls were run in the normal manner but without [19-^{3}H,^{2}H,^{1}H]- and [4-^{14}C]-3β-hydroxyandrost-5-en-17-one.

All controls and experimental incubations were run in triplicate.

Control I: H^{14}COOH, 93.4 (average of 93.8%, 95.1%, 91.3%); THOH, 76.5 (average of 76.5%, 76.7%, 76.2%); [^{3}H]estradiol, 75.9 (average of 75.6%, 78.6%, 73.5%).

Control II: H^{14}COOH, 94.0% (average of 94.0%, 93.7%, 94.2%); THOH, 82.5% (average of 81.6%, 83.8%, 82.1%); [^{3}H]estradiol, 74.7% (average of 72.2%, 75.8%, 76.1%).

Isolation of Products. The reaction mixtures, which were acidified with phosphoric acid, were frozen in liquid N_2 and lyophilized. After removal of the volatile components, water (0.5 mL) containing 0.05 mL of HCOOH was added to the flask; the mixture was frozen and lyophilized. The procedure was repeated once more with 0.5 mL of water. (A third "wash" did not contain tritium). The combined distillates were made up to 10 mL in a volumetric flask, and aliquots (100 μL each) of the solution were counted. The remaining solution was made alkaline with 1.25 M NaOH (phenolphthaleine), frozen, and lyophilized. The residue was washed with water (0.5 mL); the mixture was frozen and lyophilized. The washing operation was repeated once more. The recovered water was made up to volume (20 mL), and the resultant sodium formate was dissolved in water (10 mL) and both were counted (tritium). A portion of the sodium [^{3}H]formate was converted into benzyl formate[29] and aliquots were purified by HPLC. The peak of [^{3}H]benzyl formate retained tritium, and it coincided with the peak of authentic benzyl formate.

Preparation of Benzyl Formate. Aqueous 5% NaOH (1 mL) and mercuric oxide (50 mg) were added to a solution of benzylhydrazone (100 mg) in ether (5 mL). The mixture was shaken for several minutes and left at room temperature for 1 h. The phases were allowed to separate, and the ether phase containing phenyldiazomethane was stored in the refrigerator.

The isolated dry sodium formate was taken up in MeOH (30 μL) and then 1 M HCl (10 μL) was added, and this was followed by immediate addition of the above phenyldiazomethane solution (35 μL). Aliquots of the ester solution were analyzed by HPLC.

Isolation of Estrogens. The residue of the initial lyophilization was diluted with water (5 mL), and the steroids were extracted with ethyl acetate (EA) (4 × 5 mL). The EA extract was washed with 1.25 M NaOH (4 × 5 mL) and the obtained alkaline solution was made acidic (pH 1–2) with 10% HCl. The phenols were recovered with EA (4 × 5 mL), washed, dried, and concentrated to a residue. The residue was dissolved in ethanol (1 mL), sodium borohydride (2 mg) was added, and the mixture was stirred at room temperature for 1 h. The solution was acidified (dilute AcOH), water added, and the "estradiol" recovered in the conventional manner with EA. The EA extract was concentrated and the residue dissolved in EtOH (100 μL) and submitted to HPLC purification. The estradiol peak (18-min retention time) was collected and counted.

Acknowledgment. We are indebted to Dr. Chiu C. Chang (Postdoctoral Fellow, 1980–1981) and Dr. Karl H. Bush (Postdoctoral Fellow, 1982–1983) for the initial studies of the Kuhn–Roth oxidation and chirality determination of the derived acetic acid. We thank Dr. Thomas B. Pokoly and the staff of the Department of Obstetrics and Gynecology of the Memorial Hospital, Worcester, MA, for providing human placentae. We thank Prof. Heinz G. Floss, Ohio State University, for the chirality determination of the acetate (F = 49) derived from the "second" sample of (19*RS*)-3β-hydroxyandrost-5-en-17-one.

Registry No. Aromatase, 9039-48-9; deuterium, 7782-39-0; tritium, 10028-17-8; (19*R*)-[19-^{3}H,^{2}H,^{1}H]-3β-hydroxyandrost-5-en-17-one, 87711-63-5; (19*S*)-[19-^{3}H,^{2}H,^{1}H]-3β-hydroxyandrost-5-en-17-one, 87711-64-6.

(27) Akhtar, M.; Calder, M.; Smith, T.; Wright, J. N. *Biochem. J.* **1980**, *185*, 411.

(28) Norman, K. K. *Biochem. J.* **1909**, *4*, 127. Layne, E. "Methods in Enzymology"; Colowick, S. P., Kaplan, N. O., Eds.; Academic Press: New York, 1957; Vol. 3, p 447.

(29) Corina, D. L. *Anal. Biochem.* **1977**, *80*, 639.