Evidence of the Direct Aromatization of Testosterone and Different Aromatization Sites for Testosterone and Androstenedione in Human Placental Microsomes†

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ABSTRACT: The purposes of this study are the examination of the direct aromatization of testosterone and the determination of whether testosterone and androstenedione are aromatized at the same enzymic site in human placenta. A mixture of [17α-^{3}H]- and [4-^{14}C]testosterone was incubated with a preparation of human placental microsomes and NADPH. The incorporation of ^{3}H into 17β-estradiol in 64% yield and the retention of the ^{3}H:^{14}C ratio demonstrated the direct aromatization of the 17β-hydroxyandrogen. To determine if testosterone and androstenedione are aromatized at the same enzymic site, mixtures of [1α,2α-^{3}H]testosterone and [4-^{14}C]-androstenedione were incubated in 1:1, 5:1, and 1:5 molar ratios and in enzyme-saturating amounts with human placental microsomes and an NADPH-generating system. The time course of substrate depletion and estrogen formation was measured after purification by paper chromatography and two-dimensional thin-layer chromatography, yield correction by Kober measurement and crystallization to constant specific activity. Only in one of these incubations (1:5 testosterone:androstenedione) was the kinetics of [^{3}H]- and [^{14}C]-17β-estradiol formation consistent with competitive aromatization. Subsequent initial velocity measurements of the inhibition of the aromatization of one androgen by the other androgen, presented in the form of Dixon and Lineweaver–Burk plots, showed that androstenedione inhibited testosterone aromatization, but testosterone was much less effective in inhibiting androstenedione aromatization. However, testosterone was aromatized at one-third the rate of androstenedione aromatization when coincubated with androstenedione. Although these data do not exclude androgen aromatization from a single enzymic site with androstenedione as the preferred substrate, a multisite hypothesis for aromatization is more consistent with the data. Consequently we propose one site (A site) at which androstenedione is aromatized exclusively and a second site (T site) for the competitive aromatization of both androgens. Sodium chloride (1.2 M) stimulated testosterone aromatization by 31.5% while inhibiting androstenedione aromatization by 13.4%.

Aromatase is operationally defined as the "enzyme" which converts androgenic molecules to estrogens. Although aromatase activity is found in several tissues of the human body, a particularly rich source is the microsomal fraction of human term placenta (Ryan, 1959). A considerable effort has been made in determining the intermediates and mechanism of estrogen biosynthesis (Akhtar and Skinner, 1968; Fishman *et al.*, 1969; Meigs and Ryan, 1971; Menini and Engel, 1967; Milewich and Axelrod, 1972; Osawa, 1973; Wilcox and Engel, 1965), but little is known about substrate specificities for aromatization at the active site or about characteristics of the membrane-bound multienzyme system. Of the two principal androgens, androstenedione and testosterone, only androstenedione is unequivocally accepted in the literature as a substrate for aromatase. There is conflicting evidence regarding the aromatization of testosterone without androstenedione as an obligatory intermediate. Bolté *et al.* (1964) have presented *in vivo* evidence and Ryan (1959) and Osawa (1973) *in vitro* evidence for the direct aromatization of testosterone. On the other hand, Baulieu *et al.* (1963) and Menini and Engel (1967) suggest that testosterone aromatization may proceed solely through androstenedione using 17β-hydroxyandrogen dehydrogenase activity also present in human placenta. High levels of 17β-hydroxyandrogen dehydrogenase in the latter studies may have obscured the direct aromatization of testosterone. To the best of our knowledge, no simultaneous aromatase incubations using these two principal androgens have been done to determine the interaction among the substrates and the aromatase active site during aromatization.

The purposes of this study are the investigation of the direct aromatization of testosterone in a preparation of human placental microsomes with low 17β-hydroxyandrogen dehydrogenase activity and, having observed that, the determination of whether testosterone and androstenedione, when coincubated, compete for the same aromatization site or whether different enzymic sites are used.

Materials and Methods

Microsomal Preparation. The low-speed supernatant of fresh human term placenta was obtained by the procedure of Ryan (1959). The pellet obtained after centrifugation of this low-speed supernatant at 100,000*g* for 1 hr was suspended in distilled water, lyophilized, and stored at −20°. The aromatase activity remained stable in this condition for at least 2 years. Microsomal protein was measured by the procedure of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Steroid Substrates. [1α,2α-^{3}H]Testosterone (0.95 Ci/mmol) was produced by tris(triphenylphosphine)rhodium chloride catalytic reduction of 17β-hydroxy-1,4-androstadien-3-one with tritium (Osawa and Spaeth, 1971). [1α,2α-^{3}H]androstenedione (0.91 Ci/mmol) was synthesized from [1α,2α-^{3}H]testosterone by Jones oxidation and recrystallized from acetone–

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hexane. [4-^{14}C]Androstenedione (60 Ci/mol) and [4-^{14}C]testosterone (58 Ci/mol) were obtained from New England Nuclear Corp. and Amersham Searle, Inc., respectively. [17α-^{3}H]Testosterone (20 Ci/mol) was synthesized from androstenedione by [^{3}H]sodium borohydride reduction in 95% ethanol at 2° and recrystallized twice from aqueous ethanol. The radiochemical purity was found to be 96% by recrystallization with authentic testosterone. The nonspecific ^{3}H labeling of this compound, determined by Jones oxidation to androstenedione and recrystallization to constant specific activity, accounted for less than 1% of the radioactivity. The other [^{3}H]- and [^{14}C]androgens were checked for purity by paper chromatography in a Zaffaroni system using formamide as the stationary phase and hexane–benzene (1:1, v/v) as the mobile phase.

Aromatase Incubations. The androgen substrates were dissolved in 95% ethanol and mixed with 1 drop of propylene glycol in 25-ml erlenmeyer flasks. The amounts of substrate incubated are given in the figure legends. The ethanol was evaporated from the incubation flasks by a stream of nitrogen. The lyophilized microsomal preparation was homogenized in 0.067 M phosphate buffer (pH 7.2) at 2°. NADPH, either alone or with a generating system, was dissolved in phosphate buffer and added to the incubation flask. Both the incubation flask with substrate and NADPH and the microsomal homogenate were prewarmed to 37° for 2 min before the enzyme incubation was initiated by adding the microsomal homogenate to the flask. The incubation was carried out at 37° with shaking in air.

INCUBATION OF [4-^{14}C,17α-^{3}H]TESTOSTERONE. [17α-^{3}H]-Testosterone (61.4 nCi; 2.9 nmol), [4-^{14}C]testosterone (5.7 nCi; 0.07 nmol), androstenedione (3.5 nmol), 17β-estradiol (110 nmol), and estrone (85 nmol) were incubated with NADPH (4.3 μmol) and microsomal protein (5.4 mg) in a total volume of 2.75 ml at 37°. The incubation was terminated after 15 min with 5 ml of ethyl acetate saturated with buffer and a 95% ethanol solution containing 170 nmol each of testosterone and androstenedione, 660 nmol of 17β-estradiol, and 425 nmol of estrone. The amounts of 17β-estradiol and estrone remaining after purification were determined by the Kober procedure (Osawa and Slaunwhite, 1970) and used to correct for the overall yield.

KINETICS OF AROMATIZATION. Each flask contained substrate, 7.2 μmol of NADPH, 119 μmol of glucose 6-phosphate, 28.6 units of glucose-6-phosphate dehydrogenase, and 12 mg of microsomal protein in 5.5 ml of phosphate buffer. Aliquots of 0.5 ml were taken at various times and transferred to tubes containing 185 nmol of estrone, 173 nmol each of testosterone and androstenedione, and 735 nmol of 17β-estradiol. The amount of 17β-estradiol remaining after purification was determined by the Kober procedure (Osawa and Slaunwhite, 1970) and used to correct for the overall yield.

INITIAL VELOCITY OF AROMATIZATION. Each flask contained substrate, 2.4 μmol of NADPH, and 3.4 mg of microsomal protein in 1 ml of phosphate buffer. After 2-min incubation, unless indicated otherwise, 0.4 ml of 95% ethanol, containing 185 nmol each of estrone and 17β-estradiol, 173 nmol each of testosterone and androstenedione, 3000 dpm of [^{14}C]17β-estradiol, and 3000 dpm of [^{14}C]androgen (same androgen as that labeled with ^{3}H during the incubation), was added to the flask, followed immediately by 5 ml of ethyl acetate saturated with phosphate buffer. The amounts of radioactively labeled androgen substrate and 17β-estradiol recovered after the incubation were corrected for purification yields using the following equation: $(T)(C_0/S_0)$ = amount of 17β-estradiol produced or androgen recovered in nmol, where T is the ^{3}H:^{14}C ratio of the androgen or 17β-estradiol isolated from the incubation, C_0 is the number of disintegrations per minute of [^{14}C]androgen or 17β-estradiol added with the carrier mixture after the incubation, S_0 for recovered androgen is the specific activity in disintegrations per minute per nanomole of the [^{3}H]androgen substrate used in the incubation, and S_0 for the 17β-estradiol produced is the specific activity of the [^{3}H]androgen substrate corrected for the [1α,2α-^{3}H]retention in aromatization (Osawa and Spaeth, 1971). The initial velocity of androgen utilization was calculated by subtracting the amount of androgen recovered from the incubation from the amount incubated and dividing the result by the time of the incubation. The initial velocity of androgen aromatization was obtained by dividing the amount of 17β-estradiol recovered from the incubation by the time of the incubation since the amount of estrone and other estrogens produced under these conditions is negligible. Control experiments showed negligible androgen utilization and aromatization at zero time. Under these experimental conditions the rate of aromatization is constant up to 80–85% of the 17β-estradiol produced or androgen utilized, which includes all of the data reported for initial velocity measurements.

Processing of the Incubation Mixture. Immediately after the carrier mixture was added, each sample was extracted three times with 5 ml of ethyl acetate and the organic phase was washed once with water. The ethyl acetate extract was evaporated with a stream of nitrogen and the residue was dissolved in methanol. The methanol solution was applied to a 3-cm wide paper chromatography strip utilizing Whatman No. 1 paper with formamide as the stationary phase and benzene–hexane (1:1, v/v) as the mobile phase. Development was carried out for 3.5 hr at room temperature. Positions of the carrier testosterone and androstenedione on the paper strip were identified visually by the areas of ultraviolet (uv) absorption. The positions of carrier 17β-estradiol and estrone were located by staining a 1-mm wide strip cut from the edge of the paper strip with a 1:1 mixture of 1% $FeCl_3$–1% $K_3Fe(CN)_6$. All androgen and estrogen areas were subsequently cut from the paper strip and eluted with methanol. The average recoveries of the substrate androgens and product estrogens relative to the incubated radioactivity were 90% after organic extraction and 75% after paper chromatography. Each recovered 17β-estradiol fraction was applied to a thin-layer chromatographic plate (0.25 mm, 20 × 20 cm, Analtech) and developed twice in the first direction with cyclohexane–ethyl acetate (2:1, v/v) and once in the second direction with chloroform–methanol (95:5, v/v) at room temperature. The 17β-estradiol area was identified both by the area of uv absorption and by the position of simultaneously applied standards. This area was scraped from the plate and eluted with methylene chloride–ethanol (1:1, v/v). The average recovery of carrier 17β-estradiol after the combined separation steps of organic extraction and paper and thin-layer chromatography was 55%. In early experiments, the androgens and estrogens obtained after these separation procedures were crystallized to constant specific activity. The results after these crystallizations were qualitatively identical and quantitatively within ±10% of the results obtained before crystallization. Therefore, this crystallization step was used only occasionally in later experiments to ascertain that this condition was maintained.

Radioactivity was measured as described previously (Osawa and Spaeth, 1971). The conversion to disintegration per minute was made using quenched standards.

TABLE I: Radioactivity in Androgens and Estrogens Isolated from Aromatization of [4-^{14}C,17α-^{3}H]Testosterone.

Compound	% ^{3}H[b]	% ^{14}C[b]	^{3}H:^{14}C[a]
17β-Estradiol[c]	15.4	9.2	17.8
Estrone[c]	0.0	5.3	<0.03
Testosterone[d]	76	74.5	11.0
Androstenedione[d]	0.2	5.8	0.32

[a] Initial ^{3}H:^{14}C of 10.8 with 136,000 dpm of ^{3}H and 12,650 dpm of ^{14}C. [b] Based on radioactivity recovered in organic phase after ethyl acetate extraction (aqueous phase contained <4% of this radioactivity). [c] After purification by paper chromatography and two-dimensional tlc and corrected for yield by the Kober procedure. [d] After purification by paper chromatography. No yield correction was made.

Experimental Design. DIRECT AROMATIZATION OF TESTOSTERONE. To show that androstenedione is not an obligatory intermediate for testosterone aromatization, [17α-^{3}H]testosterone was prepared as a substrate for aromatization. Isolation of [^{3}H]17β-estradiol from the incubation is required to show that the 17β-hydroxy steroid substrate is not converted to the 17-keto steroid as a necessary prerequisite for aromatization. Pools of unlabeled androstenedione, estrone and 17β-estradiol were included in the incubation to (a) minimize the loss of ^{3}H when 17β-estradiol is converted to estrone by 17β-hydroxyestrogen dehydrogenase activity and (b) estimate the extent of 17β-hydroxyandrogen dehydrogenase activity by the recovery of radioactivity in the androstenedione pool. The duration of the incubation was short (15 min) so that less than 30% of the testosterone was aromatized.

AROMATIZATION SITES OF TESTOSTERONE AND ANDROSTENEDIONE. To determine if testosterone and androstenedione compete for the same aromatization site, two types of experiments were carried out. First, [^{3}H]testosterone and [^{14}C]-androstenedione were coincubated in amounts that approximately saturate the aromatization sites. The amounts of [^{3}H]- and [^{14}C]17β-estradiol produced were measured at various times until the aromatization was complete. If both substrates compete for the same site the higher affinity substrate will initially be aromatized at a greater rate, thus increasing the relative concentration of the lower affinity substrate. As aromatization proceeds, both androgens will be withdrawn from a common pool at relative rates proportional to their affinity for the site and instantaneous concentrations. Therefore, we expect that (1) changes in the rate of aromatization of one androgen will be reflected by changes in the rate of aromatization of the other androgen and (2) the [^{3}H]- and [^{14}C]17β-estradiol formed should approach their respective terminal amounts at the same time regardless of the relative initial amounts of androgen present and the relative initial rates of aromatization. Alternatively, if different sites are used, there need not be any relationship between the times these terminal amounts are reached. Success in observing this latter result, however, is dependent upon sufficiently low 17β-hydroxyandrogen dehydrogenase activity to avoid rapid equilibration of ^{3}H and ^{14}C in both androgen substrates. In this set of experiments, using a preparation of human placental microsomes with low 17β-hydroxyandrogen dehydrogenase activity, several molar ratios of [^{3}H]testosterone and [^{14}C]-androstenedione were coincubated and the kinetics of androgen utilization and estrogen formation were measured.

TABLE II: Crystallization of 17β-Estradiol Obtained from Aromatization of [4-^{14}C,17α-^{3}H]Testosterone.

	Specific Activity (dpm/μmol)		
Crystallization[a]	^{3}H	^{14}C	^{3}H:^{14}C
First—crystals	254	14.5	17.4
Mother liquor	256	14.7	17.4
Second—crystals	260	15.4	16.9
Third—crystals	264	15.6	17.6

[a] Authentic 17β-estradiol was added to the labeled 17β-estradiol after paper chromatography and tlc purification, and recrystallized from aqueous ethanol.

In the second type of experiment, the initial velocity of [^{3}H]17β-estradiol production was measured during the aromatization of various concentrations of one [^{3}H]androgen coincubated with several concentrations of the other unlabeled androgen. The data were analyzed by the Dixon plot and the Lineweaver–Burk plot to determine if the unlabeled androgen acts as a competitive inhibitor of the aromatization of the [^{3}H]androgen.

Results

Direct Aromatization of Testosterone. The results of the incubation of [4-^{14}C,17α-^{3}H]testosterone are shown in Table I. Of the 25.5% ^{14}C label unrecovered as testosterone after the 15-min incubation, about one-third was isolated as 17β-estradiol, one-fifth as estrone, and one-fifth as androstenedione. Incorporation of about two-thirds of the ^{3}H unrecovered as testosterone into [^{3}H]17β-estradiol and the retention of this [^{3}H]17β-estradiol through crystallization to constant specific activity, as shown in Table II, demonstrate the direct aromatization of testosterone in this microsomal preparation. A primary isotope effect of 17β-hydroxyestrogen dehydrogenase, suggested by the increased ^{3}H:^{14}C ratio of the isolated 17β-estradiol relative to the initial ratio in testosterone and by the fact that the sum of [^{14}C]17β-estradiol and [^{14}C]-estrone approximately equals the amount of [^{3}H]17β-estradiol, has been described by Adams *et al.* (1965) and Karavolas *et al.* (1969).

Kinetics of Androgen Aromatization. The results of coincubations of [^{3}H]testosterone and [^{14}C]androstenedione in different molar ratios with placental microsomes are presented in Figures 1–3. Depletion of substrates and the kinetics of testosterone–androstenedione interconversion when [^{3}H]-testosterone and [^{14}C]androstenedione are incubated in a 1:1 molar ratio are shown in Figure 1a. The low level of androgen interconversion provides evidence for low 17β-hydroxyandrogen dehydrogenase activity relative to aromatase activity. The formation of [^{3}H]- and [^{14}C]17β-estradiol is shown in Figure 1b. Estrone comprised less than 3% of the initial radioactivity throughout the incubation. [^{14}C]Androstenedione is depleted sooner than [^{3}H]testosterone and [^{14}C]17β-estradiol is formed more rapidly and reaches the terminal amount earlier than [^{3}H]17β-estradiol. Reversing the radioactive labels on the androgen substrates had no effect on the results. The ^{3}H:^{14}C ratios for 17β-estradiol, estrone, and androstenedione during the incubation are shown in Figure 1c. The ^{3}H:^{14}C ratio for androstenedione is less than that for estrone or 17β-estradiol until the androstenedione aromatization is about 90% complete after 30 min. This re-

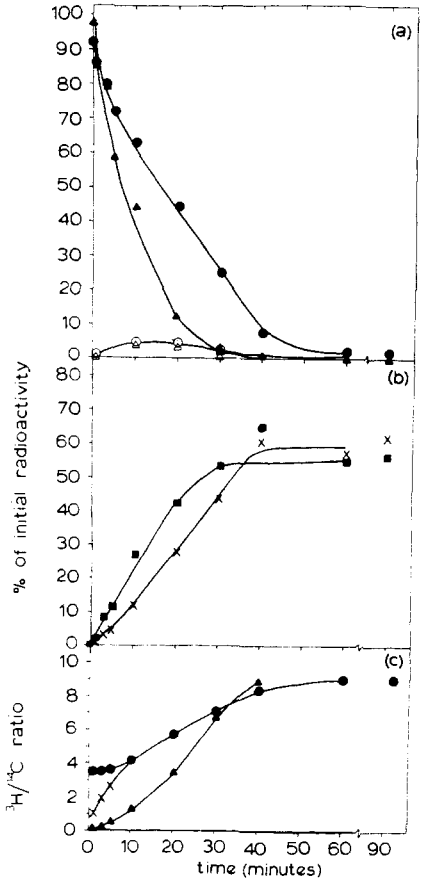

FIGURE 1: Simultaneous aromatization of testosterone and androstenedione incubated in a 1:1 molar ratio. [^{3}H]Testosterone (4.3 μCi; 6.9 nmol) and [^{14}C]androstenedione (0.4 μCi; 7 nmol) were incubated with a placental microsomal homogenate and a NADPH-generating system in air. Aliquots were removed at various times and processed as described. The percentage of the initial radioactivity incubated is recorded on the ordinate: (a) (●) [^{3}H]testosterone, (▲) [^{14}C]androstenedione, (○) [^{14}C]testosterone, (△) [^{3}H]-androstenedione; (b) (■) [^{14}C]17β-estradiol, (×) [^{3}H]17β-estradiol; (c) (●) ^{3}H:^{14}C ratio of 17β-estradiol, (×) ^{3}H:^{14}C ratio of estrone, (▲) ^{3}H:^{14}C ratio of androstenedione. The theoretical final value of the ^{3}H:^{14}C ratio of the estrogens is 9.3 (Osawa and Spaeth, 1971).

sult is consistent with the earlier result that androstenedione is not an obligatory intermediate for testosterone aromatization since if it were the ^{3}H:^{14}C ratio for estrone and 17β-estradiol would be equal to or less than that of androstenedione.

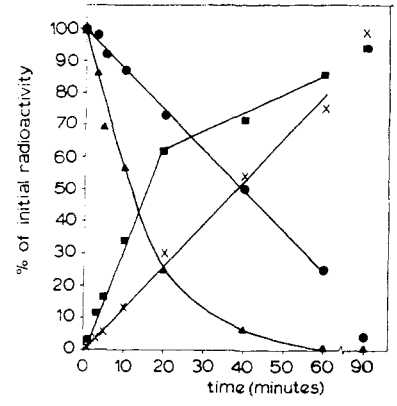

FIGURE 2: Simultaneous aromatization of testosterone and androstenedione incubated in a 5:1 molar ratio. [^{3}H]Testosterone (4.0 μCi; 34.6 nmol) and [^{14}C]androstenedione (0.39 μCi; 7 nmol) were incubated as described in Figure 1: (●) [^{3}H]testosterone; (▲) [^{14}C]-androstenedione; (■) [^{14}C]17β-estradiol; (×) [^{3}H]17β-estradiol.

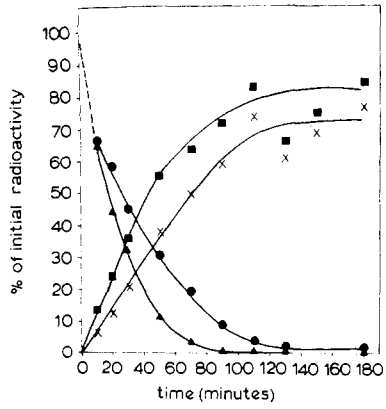

FIGURE 3: Simultaneous aromatization of testosterone and androstenedione incubated in a 1:5 molar ratio. [^{3}H]Testosterone (4.3 μCi; 6.9 nmol) and [^{14}C]androstenedione (0.44 μCi; 34.8 nmol) were incubated as described in Figure 1: (●) [^{3}H]testosterone; (▲) [^{14}C]-androstenedione; (■) [^{14}C]17β-estradiol; (×) [^{3}H]17β-estradiol.

[^{3}H]Testosterone and [^{14}C]androstenedione were then co-incubated in a 5:1 molar ratio, respectively, with placental microsomes. The results, plotted as the per cent of the initial radioactivity, are shown in Figure 2. After about 20 min a reduction in the rate of [^{14}C]17β-estradiol formation was observed. However, no change was observed in the rate of [^{3}H]-17β-estradiol formation over the duration of the experiment. The percentages of androgen interconversion and estrone synthesized were similar to that found in the 1:1 incubation.

When [^{3}H]testosterone and [^{14}C]androstenedione were co-incubated in a 1:5 molar ratio, the androgen interconversion and kinetics of estrone formation were again similar to those shown for the 1:1 incubation. The kinetics of androgen utilization and 17β-estradiol formation are shown in Figure 3. Although the total time required to aromatize both androgens is longer than that in the 1:1 incubation (Figure 1), a reduction in the rate of both [^{3}H]- and [^{14}C]17β-estradiol formation as well as the terminal amount of both labels in 17β-estradiol are reached at about the same time.

Initial Velocity of Androgen Aromatization. The inhibition of aromatization of [^{3}H]androstenedione by testosterone and of [^{3}H]testosterone by androstenedione is presented in the form of a Dixon plot in Figure 4. Testosterone (69 nM) produces little, if any, inhibition of the aromatization of 52.5 nM [^{3}H]androstenedione. This result is consistent with the

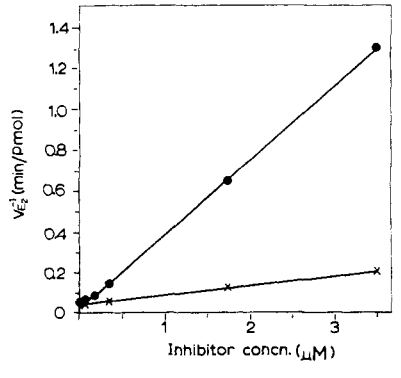

FIGURE 4: Inhibition of aromatization of one androgen in the presence of the other androgen. The initial rate of [^{3}H]17β-estradiol produced from [^{3}H]androgen incubated with placental microsomes and NADPH in the presence of increasing amounts of the other nonradioactively labeled androgen is presented in the form of a Dixon plot: (×) [^{3}H]androstenedione (0.056 μCi; 52.4 pmol) with nonradioactive testosterone; (●) [^{3}H]testosterone (0.54 μCi; 55.5 pmol) with nonradioactive androstenedione.

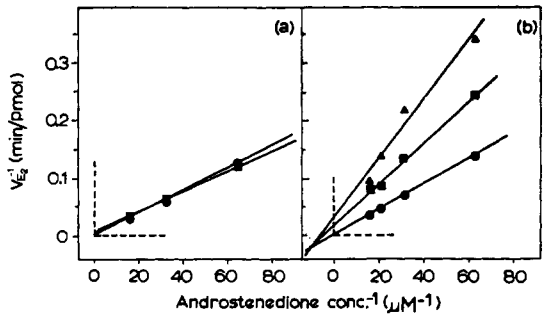

FIGURE 5: Double-reciprocal plot of the aromatization of androstenedione in the presence of testosterone. Several concentrations of [^{3}H]androstenedione (0.91 Ci/mmol) were incubated as described with several concentrations of testosterone. The initial rates of [^{3}H]17β-estradiol production were measured. The line through each set of data points is a linear least-squares fit to the data: (a) (●) no testosterone, (■) 69.4 nM testosterone; (b) (●) no testosterone, (■) 866 nM testosterone, (▲) 1.73 mM testosterone.

report by Schwarzel *et al.* (1973) that the 17-keto group is more effective than 17β-ol as an inhibitor of androstenedione aromatization. However, 70 nM androstenedione is effective as an inhibitor of the aromatization of 55.5 nM [^{3}H]testosterone. At high concentrations of the unlabeled androgen (>350 nM), each androgen inhibits the aromatization of the other androgen and again androstenedione is considerably more effective as an inhibitor of testosterone aromatization. The nature of this inhibition of aromatization was examined in more detail by data presented in the form of a Lineweaver–Burk plot. The interpretation of these data is based on a linear least-squares fit. In Figure 5 the double-reciprocal plots of the rate of formation of [^{3}H]17β-estradiol for several concentrations of [^{3}H]androstenedione with and without testosterone are shown. The apparent K_m and V_{max} for androstenedione aromatization are about 5.3×10^{-7} M and 76.5 pmol/min per mg. In Figure 5a, 69 nM testosterone had no apparent effect on the initial velocity of aromatization of androstenedione. Large amounts of testosterone (>0.8 μM) did inhibit the aromatization of androstenedione (Figure 5b). A strict interpretation of the least-squares fit to the data in Figure 5b suggests noncompetitive inhibition, although the difference between competitive and noncompetitive inhibition could be obscured by experimental error.

Results of similar experiments with [^{3}H]testosterone are presented in Figures 6 and 7. The reciprocal of the initial rate of [^{3}H]testosterone utilization and the reciprocal of the initial rate of formation of [^{3}H]17β-estradiol are shown as a function of the reciprocal of testosterone concentration in Figures 6a and 7a, respectively. From these data, the apparent K_m and V_{max} for testosterone aromatization are about 1×10^{-7} M and 10.3 pmol/min per mg and for testosterone utilization are about 2.8×10^{-7} M and 24.7 pmol/min per mg. Figures 6b,c and 7b,c show the decreased velocity of testosterone utilization and aromatization as the androstenedione concentration is increased. [^{3}H]Testosterone utilization and aromatization are competitively inhibited by comparable amounts of androstenedione (see Figures 6d and 7d).

In summary, the preceding results are outlined as follows. (1) Androstenedione is not an obligatory intermediate for testosterone aromatization (see Table I and Figures 1, 2, 3, and 7). (2) Although testosterone is a relatively poor inhibitor of androstenedione aromatization (see Figures 4 and 5), androstenedione is a relatively good inhibitor of testosterone aromatization (see Figures 4, 6, and 7). (3) The rate of aromatization of 63 nM [^{3}H]androstenedione (26.8 pmol/min)

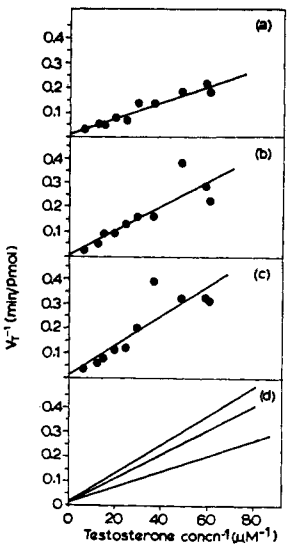

FIGURE 6: Double-reciprocal plot of the utilization of testosterone in the presence of androstenedione. Several concentrations of [^{3}H]testosterone (0.95 Ci/mmol) were incubated as described with several concentrations of androstenedione. The initial rates of testosterone utilization were measured. Six out of 10 data points in each set are from 1.5-min incubations. The line through each set of data points is a linear least-squares fit to the data: (a) no androstenedione; (b) 70 nM androstenedione; (c) 140 nM androstenedione; (d) the lines from each data set are redrawn for comparison.

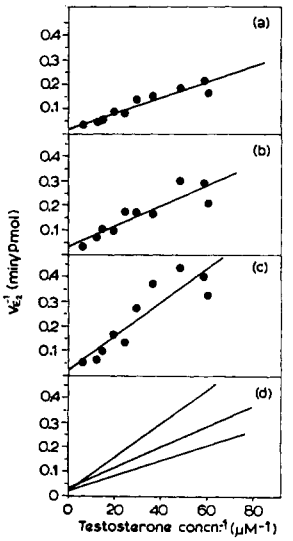

FIGURE 7: Double-reciprocal plot of the aromatization of testosterone in the presence of androstenedione. These data were taken from the experiment described in Figure 6 except that the initial rates of 17β-estradiol production were measured: (a) no androstenedione; (b) 70 nM androstenedione; (c) 140 nM androstenedione; (d) the lines from each data set are redrawn for comparison.

was not appreciably affected by the simultaneous presence of 69 nM testosterone (taken from two data points in Figure 5a). (4) When incubated under identical conditions as those in (3), the rate of aromatization of 68.5 nM [^{3}H]testosterone, when coincubated with 61 nM androstenedione, was 9 pmol/min (taken from one data point in Figure 7b).

Effect of NaCl on the Coaromatization of Testosterone and Androstenedione. Several compounds[1] which are reported to inhibit aromatization were incubated with our microsomal

[1] Flavine mononucleotide, flavine adenine dinucleotide, metapyrone, elipten phosphate, and SKF-525-A, all at 10^{-3} M; iodoacetamide and *p*-hydroxymercuribenzoate at 10^{-4} M.

preparation to determine if a differential inhibition of testosterone and androstenedione aromatization could be observed. None of these compounds showed a differential effect. Only when NaCl was included in the incubation medium was a differential effect observed. [^{3}H]Testosterone (1.27 mM) and [^{14}C]androstenedione (1.23 mM) were coincubated with 3.3 mg of protein, 1.2 mM NADPH, and 1.2 M NaCl in 1 ml of buffer at 37° for 6 min. In six control incubations, the means and standard deviations of the initial radioactivity isolated in the 17β-estradiol fraction were 13.0 ± 0.55% as ^{3}H and 29.0 ± 0.83% as ^{14}C. When NaCl was included in six incubations, 17.1 ± 0.64% was isolated in 17β-estradiol as ^{3}H and 25.2 ± 1.4% as ^{14}C. The means of the [^{3}H]-and [^{14}C]17β-estradiol for the salt *vs.* control incubations are different to all significance levels >0.001. Therefore, NaCl stimulated testosterone aromatization by 31.5% while inhibiting androstenedione aromatization by 13.4%. NaCl also inhibited the 17β-hydroxyandrogen dehydrogenase activity by about 50% over the control level.

Discussion

Direct Aromatization of Testosterone. The quantitative retention of the 17α-^{3}H label in 17β-estradiol formed from [17α-^{3}H]testosterone provides conclusive evidence for the direct aromatization of testosterone. This result is in qualitative agreement with that of Bolté *et al.* (1964) who performed a similar experiment with perfused human placenta *in situ*. Quantitatively, their evidence is not as strong as the evidence presented here since they found less than 0.5% of the ^{3}H from testosterone in 17β-estradiol and apparently lost more than 99.5% of the 17α-^{3}H by other metabolic processes. A transhydrogenase transfer of ^{3}H from testosterone to 17β-estradiol through reduction of estrone (Pollow and Pollow, 1972) is considered insignificant due to the low 17β-hydroxyandrogen dehydrogenase activity in these microsomes and to the large pool of exogenous NADPH included in the incubation.

Further evidence for the direct aromatization of testosterone taken from the kinetics of simultaneous androgen aromatization is (a) the inequality of the ^{3}H:^{14}C ratio for androstenedione and the estrogens until the aromatization of androstenedione is about 90% complete as shown in Figure 1c, (b) the activation of testosterone aromatization while androstenedione aromatization and androgen interconversion were inhibited by 1.2 M NaCl, and (c) the small percentage ($\simeq$5%) of [^{3}H]androstenedione observed when [^{3}H]testosterone and [^{14}C]androstenedione were simultaneously coincubated.

Enzymic Site of Androgen Aromatization. It is evident from the data reported here that both testosterone and androstenedione serve as substrates for aromatase and that, when coincubated in equimolar amounts, androstenedione is aromatized more rapidly. Therefore, if testosterone and androstenedione compete for a single aromatization site, androstenedione must have a higher affinity for that site. Alternatively, the apparent preferential androstenedione aromatization may be explained by a multisite hypothesis with more aromatization sites available for androstenedione than for testosterone. Some of the evidence obtained is incompatible with our expectations for a single aromatization site. These are as follows. (1) In the kinetics of simultaneous aromatization of the two androgens (Figures 1–3), only the data presented in Figure 3 are strictly consistent with our expectation for competitive aromatization as explained in the experimental design section. Particularly, the distinct decrease in the rate of androstenedione aromatization while the rate of testosterone aromatization remains constant (Figure 2) is difficult to reconcile with competitive aromatization from a single aromatization site. (2) In the initial velocity of aromatization experiments, testosterone, when coincubated with androstenedione in concentrations that had no observable effect on the rate of androstenedione aromatization (up to 70 nM), was aromatized at one-third the rate of androstenedione aromatization.

Because of these difficulties in interpreting the data using a single aromatization site, we tentatively propose a multisite model composed, in its simplest form, of two sites. One site, the A site, aromatizes androstenedione rather exclusively. The other site, the T site, competitively aromatizes testosterone and androstenedione. The results from the coincubation of [^{3}H]testosterone and [^{14}C]androstenedione in several molar ratios are consistent with this two-site model. When both androgens were incubated in a 1:1 molar ratio, androstenedione was aromatized from both sites and competitively inhibited the aromatization of testosterone. Thus testosterone was aromatized at a lower rate than if androstenedione were not present and testosterone aromatization did not terminate until the androstenedione aromatization was complete. At a 5:1 testosterone to androstenedione ratio, androstenedione was aromatized preferentially at the A site and, because of the lower amounts of androstenedione, the rate of androstenedione aromatization decreased earlier than the rate of testosterone aromatization, as observed in Figure 2. When testosterone and androstenedione were present in a 1:5 molar ratio, respectively, androstenedione was aromatized from both sites, competitively inhibiting the aromatization of testosterone at the T site. Therefore, testosterone aromatization could not terminate until androstenedione was depleted.

This two-site model can also account for the apparent lack of inhibition of androstenedione aromatization by testosterone under conditions in which a significant amount of testosterone is aromatized. In this model, testosterone aromatization from the T site might be expected to decrease the rate of androstenedione aromatization at the T site due to competitive inhibition. However, the overall rate of androstenedione aromatization may be unaffected if the rate of aromatization at the A site is increased by an increase in free androstenedione concentration due to the displaced androstenedione from the T site.

The values for apparent K_m and V_{max} reported here must be accepted very cautiously since these values represent the net effect of several enzymic reactions and a crude microsomal preparation was the source of enzymic activity. These values may represent a more physiological situation than values obtained from a purified enzyme preparation (Wiener and Allen, 1967). Another problem of the relatively impure microsomal preparation of aromatase is that the interpretation of the results, based on the assumption that the steroid concentrations measured are at least directly proportional to the concentrations available to the active sites, may be in error. Using mouse testes homogenates, Matsumoto and Samuels (1969) found that the relative concentrations of progesterone and 17α-hydroxyprogesterone bound to the microsomes differed from that in the medium and therefore warned against "studies of relative rates of reaction involving microsomally bound enzymes." We are currently attempting to further purify aromatase so that these studies can be repeated on a more purified preparation.

These proposed sites of androgen aromatization need not be located on separate proteins; we have no data yet to suggest either a single protein or two separate proteins. The major difference between the sites themselves must lie in the region complementary to the 17 position of the steroid. Different electronic states of the oxygen atom in alcohol and ketone,

which presumably engages in hydrogen bonding with the binding site, or simply different volumes of the 17-alcohol and ketone due to the bulkier sp^3 hybridization of the C-17 in testosterone compared with sp^2 hybridization of the C-17 in androstenedione may influence the binding of testosterone and androstenedione to the two different aromatization sites. If the size of the group at the C-17 influences the binding at the aromatization sites, then the T site may have a larger volume complementary to the 17 position to accommodate both testosterone and androstenedione. The corresponding position in the A site may be only large enough for the 17-keto group, thereby excluding testosterone. Two substrate-specific Δ^5-3-ketosteroid isomerases from bovine adrenals have been reported (Ewald *et al.*, 1964), in which the substrates differ only in the group attached to C-17.

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Added in Proof

Samuels and Matsumoto (1974) recently reported that 17-hydroxyprogesterone, androstenedione, and testosterone probably diffuse relatively freely between mouse testis microsomes and the suspension medium.

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