In the stallion testis, aromatase activity was localized in the microsomal fraction. Androgen aromatization occurred through the loss of 1,2,6-hydrogen atoms and appeared to involve free sulfhydryl groups. A single enzyme system seemed to aromatize androgen and norandrogen at the same rate while having a much lower affinity for norandrogens.

Estrogen biosynthesis occurs from 4-androstenedione and testosterone via their 19-hydroxylated intermediates, the enzyme complex involved being known as the “aromatase” system (EC 1.6.2.4). C-19-Norsteroids are aromatized far less effectively than the parent C-19 methyl-steroids by human placental microsomes (1–7) or human prostate homogenates (8).

Norandrogens have been isolated from human (9) and equine follicular fluid (10, 11), from stallion urine (12), and from boar (13) and stallion testes (14) and produced in vitro by tissue from the follicular wall and corpus luteum (15, 16) of the mare, stallion testicular homogenates (17), mouse kidney slices (18), and dog adrenal gland homogenates (19). They are largely used as an anabolic agent.

The administration of esterified 19-nortestosterone reduces serum gonadotropin and testosterone concentration and results in azoospermia in men (20). However, the physiological role as sexual steroid remains unclear.

Whereas estrogens are detected in the urine of all male mammals in small amounts, they are excreted in massive amounts by the boar and stallion (21). The testicular origin of the major part of estradiol in the human, dog, and monkey has been revealed by differential concentrations of estradiol in the peripheral and spermatic veins (22). Estrogens in the stallion testis are mainly present as estrone and estradiol-3-sulfate (23), whereas in plasma the bulk of estrogens would be essentially estrone-3-sulfate (24). Stallion testis produces large amounts of estrogens in vitro (25). Androstenedione aromatization by ETMES(†) proceeds via 19-hydroxy-androst-4-ene-3,17-dione and 19-oxoandrost-4-ene-3,17-dione (26).

The significance of the presence of high levels of estrogens and particularly of estrogen sulfate in the boar and stallion is largely unknown. However, active immunization against estrogen sulfate has been revealed by differential concentrations of estradiol, or by RIA of the antisemur for estrone were 19-nortestosterone, 0.03%; norandrostenedione, 0.2%; those for estradiol were 19-nortestosterone, 0.006%; norandrostenedione ± 0.001%. The 19-nortestosterone anti-cum (cross-reactivity of 19-nortestosterone, 100%; norandrostenedione, 135%; testosterone and androstenedione < 0.05; estrone and estradiol < 0.001) was a gift of Dr. Benoit (Charbonnières, France). Chemicals and solvents of analytical grade were obtained from Sigma, Merck (Darmstadt, BDR), and Carlo Erba.

Preparation of Microsomes and Mitochondria

Tests from four stallions (2, 4, 8 years, and “aged”) were obtained from the slaughterhouse immediately after slaughtering the animals. After decapsulation, testicular tissue was dissected on ice, minced, and then frozen until use. Microsomes were isolated from testicular homogenates by differential centrifugation according to the method of Ryan (1). Homogenization was carried out with a Waring Blendor (3 × 20 s) and a potter in a 0.05 M Tris maleate, pH 7.4. buffer (buffer I) containing 0.25 M sucrose (20 vols of buffer per g of tissue). The homogenate was centrifuged at 30,000 × g for 20 min. The supernatant was then centrifuged at 230,000 × g for 60 min. Cytoplasmic residues were washed out by two additional centrifugations. After a final centrifugation in buffer I, the microsomes were diluted to 25 µg of protein/µl and stored at −25°C until required.

Mitochondria were prepared according to the Canick and Ryan procedure (28). Proteins were assessed by the Lowry et al. (29) technique using bovine serum albumin as standard.

Incubations

The activity of the aromatase preparation was assessed by measurement of 3H2O released from [1,2,3-3H]androgens, or by RIA of the estradiol and estrone formed.

Incubations were performed in 1 ml of reaction volume containing 0.5 mg of microsomal protein in buffer I and 1.5 µM androgen. The beginning of the incubation time was set at the addition of NADPH (0.3 mM). Tubes were vortexed every 3 min. All incubations were performed for 20 min at 37°C in air.

3H2O Formation—Aromatase activity was assessed according to the method of Thompson and Siiteri (30) by measuring the loss of tritium to water from 1.5 µM [1,2,3-3H]testosterone or [1,2,3-3H]androstenedione. Incubation was stopped by adding CHCl3 (1 ml). 10% charcoal activated 1.5% dextran·T70 in buffer I (500 µl) was added to 750 µl of the aqueous phase obtained by centrifugation. The mixture was allowed to sit on ice for 2 min prior to addition of 6 mg/ml of protamine sulfate (250 µl). After sedimentation (2700 × g for 10 min), the 3H2O released was assayed by measuring the radioactivity of 500 µl of supernatant in a scintillation counter.

Experimental Procedures

Chemicals

Labeled steroids, [1,2,3-3H]androstenedione (50 Ci/mmol), [1,2,3-3H]testosterone (50 Ci/mmol; α-H content unknown) were purchased from New England Nuclear, France; [1,2,3-3H]testosterone (50 Ci/mmol) (containing about 18% β-H), [2,4,6,7-3H]estradiol (95 Ci/mmol), [2,4,6,7-3H]estradiol (89 Ci/mmol), [2,4,6,7-3H]testosterone (94 Ci/mmol), [2,6,7,8-3H]testosterone (90 Ci/mmol), and [6,7-3H]nortestosterone (19 Ci/mmol) were purchased from American Corp., France. Radioimmunossay (RIA) reagents were obtained from bioMérieux (Clichy, France). The cross-reactions of the antiserum for estrone were 19-nortestosterone, 0.03%; norandrostenedione, 0.2%; those for estradiol were 19-nortestosterone, 0.006%; norandrostenedione ± 0.001%. The 19-nortestosterone antiserum (cross-reactivity of 19-nortestosterone, 100%; norandrostenedione, 135%; testosterone and androstenedione < 0.05; estrone and estradiol < 0.001) was a gift of Dr. Bénoit (Charbonnières, France). Chemicals and solvents of analytical grade were obtained from Sigma, Merck (Darmstadt, BDR), and Carlo Erba.

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Tests from four stallions (2, 4, 8 years, and “aged”) were obtained from the slaughterhouse immediately after slaughtering the animals. After decapsulation, testicular tissue was dissected on ice, minced, and then frozen until use. Microsomes were isolated from testicular homogenates by differential centrifugation according to the method of Ryan (1). Homogenization was carried out with a Waring Blendor (3 × 20 s) and a potter in a 0.05 M Tris maleate, pH 7.4, buffer (buffer I) containing 0.25 M sucrose (20 vols of buffer per g of tissue). The homogenate was centrifuged at 30,000 × g for 20 min. The supernatant was then centrifuged at 230,000 × g for 60 min. Cytoplasmic residues were washed out by two additional centrifugations. After a final centrifugation in buffer I, the microsomes were diluted to 25 µg of protein/µl and stored at −25°C until required.

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Aromatization of 19-Norandrogens by Equine Testicular Microsomes*

(Rceived for publication, July 21, 1986)

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Norandrogen Aromatization by Equine Testes Microsomes

RIA of Estrone and Estradiol—Incubations were stopped by adding 2.5 ml of ethylacetate/diethylether (1:1, v/v) (solvent I). The aqueous phase was then submitted to three extractions by 3 ml of solvent I. The pooled organic phases were evaporated to dryness (extraction yield was 95-97%) and dissolved in 4 ml of bovine albumin phosphate buffer (bioMérieux, France). Radioimmunosassays were performed as described previously (31): 100 µl of ^{3}H-labeled steroid (5 pg) and 100 µl of antisera were added to 100 µl of sample. Following equilibration (30 min at 37 °C and overnight at 4 °C) free and bound steroids were separated by dextran-coated charcoal. Nonspecific binding was calculated for each assay sample.

Thin-layer Chromatography

TLC was performed after incubations with [1α,2α-^3H]testosterone and [16β,2α-^3H]testosterone. ^{3}H_{2}O formation was measured in one set of incubates. Steroids in the second set were extracted four times by solvent I and chromatographed on 0.25-mm silica gel plates in the cyclohexane/ethylacetate system (1:1; v/v). Authentic steroids were co-chromatographed, and spots were localized under UV light. Chromatograms of incubations were scraped off in 5-mm wide strips which were eluted four times by 2.5 ml of solvent I, the radioactivity of the eluates was measured, and the results were not corrected for losses (95% recovery).

RIA of Endogenous Steroids in Microsomes

Microsomes (10 mg of protein) were extracted by 4 x 4 ml of diethyl ether for estrogen RIA or hexane/diethyl ether (4:1, v/v) for androgen RIA.

RESULTS

Experimental Conditions

Aromatization of androgen was linear up to 30 min and to a protein concentration of 0.75 mg assay (results not shown), substrate availability being the limiting factor.

Aromatase activity remained unchanged after addition of MgCl₂ and 2-mercaptoethanol (up to 100 mM) to the incubation medium and was slightly inhibited by EDTA (15% inhibition by 10 mM) and high concentrations of KCl (0% inhibition by 125 mM; 10% by 250 mM, 40% by 500 mM). Ethylene glycol had no positive effect on androgen solubility under our experimental conditions.

Enzyme Localization—Aromatase activity was found in association with the 170,000 × g precipitate (microsomes); no activity was measured in the 170,000 × g supernatant (cytosol) or in the mitochondria (Table I).

Thermosensitivity of the Aromatase System—After 30 min of preincubation at 30 °C, no loss of activity was observed, and the percentage of activity remaining at 37 °C was 67%. The enzyme was inactivated by preincubation at 45 °C (Fig. 1A). After preincubation at 37 °C for increasing time periods (Fig. 1A, inset), the remaining activity was 61% after 20 min and 40% after 55 min of preincubation. The reaction rate (Fig. 1B) increased with the reaction temperature up to 40 °C/20 min of incubation or 45 °C/5 min of incubation.

Effect of Detergent—With 2 mg of protein and 7 min of incubation, enzymatic activity was drastically inactivated by Triton X-100. ^{3}H_{2}O release was decreased by 50% with 0.5% Triton X-100 (w/v) and 90% with 1%.

Endogenous Steroids—Estrogen and androgen content of testicular microsomes are shown in Table II.

### Table I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Aromatase activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>13.1 ± 0.9</td>
</tr>
<tr>
<td>800 × g precipitate</td>
<td>7.5 ± 0.4</td>
</tr>
<tr>
<td>800 × g supernatant</td>
<td>13.5 ± 0.3</td>
</tr>
<tr>
<td>10,000 × g precipitate</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>10,000 × g supernatant</td>
<td>16.1 ± 0.3</td>
</tr>
<tr>
<td>170,000 × g precipitate</td>
<td>27.2 ± 1.5</td>
</tr>
<tr>
<td>170,000 × g supernatant</td>
<td>0.4 ± 0.05</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0.3 ± 0.02</td>
</tr>
</tbody>
</table>

* n = 6. Expressed as ^3H_2O release in pmol/min/mg of protein from [1α,2α-^3H]testosterone.

### Table II

<table>
<thead>
<tr>
<th>Age</th>
<th>Testosterone</th>
<th>Androstenedione</th>
<th>19-Norandrostenedione + norandrostenedione</th>
<th>Estradiol</th>
<th>Estriol</th>
</tr>
</thead>
<tbody>
<tr>
<td>years</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.6</td>
<td>0.2</td>
<td>0.03</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>4</td>
<td>28.7</td>
<td>1.5</td>
<td>0.4</td>
<td>28.5</td>
<td>12.6</td>
</tr>
<tr>
<td>8</td>
<td>13.4</td>
<td>18.2</td>
<td>1.9</td>
<td>44.6</td>
<td>36.9</td>
</tr>
<tr>
<td>Aged</td>
<td>41.2</td>
<td>7.9</td>
<td>1.7</td>
<td>51.7</td>
<td>35.6</td>
</tr>
<tr>
<td>16</td>
<td>2.3</td>
<td>9</td>
<td>48.4</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>
Aromatization of Androgens

Inhibition of Aromatase by Sulphydryl Reagent—10 μM sodium-p-hydroxymercurobenzoate reduced aromatization by 60%. No inhibition was observed with 1 μM, and full inhibition was obtained with 100 μM sodium-p-hydroxymercurobenzoate.

Sterespecific Elimination of Hydrogen Atoms—The estrogens formed during incubation were isolated by TLC and their 3H content compared with 3H2O release (Table III). Tritium content of aromatized androgens was calculated as 3H-estrogen + 3H2O. After incubation with [1α,2α-3H]testosterone, 3H2O contained 24% of the radioactivity of aromatized androgens. New England Nuclear indicates that 16% of the label is localized at the α position. After adequate correction, 90.5% of the radioactivity at the α position of aromatized androgens was retained in 3H2-estrogen. After incubation of [1α,2α-3H]testosterone, 37% of the radioactivity of the aromatized androgens was found in the 3H-estrogen.

The percentage of tritium of [1α,2α-3H]testosterone at the α position as determined by Reed and Ohno (32) is about 32% in the β labeled testosterone from New England Nuclear. After correction, 93% of the radioactivity in the β position of aromatized androgens was found in 3H2O.

Kinetics of Aromatization—Both androstenedione and testosterone were aromatized by testicular microsomes; the estrogens produced were essentially estrone and estradiol (Table IV). Fig. 2 (inset) shows the relation between testosterone concentrations (including microsomal endogenous testosterone) and the initial rate of aromatization. Double-reciprocal plots with a range of testosterone concentrations showed an approximate apparent K_m of 0.03 μM (Fig. 2), which is similar to the findings (0.03 μM for androstenedione) of Miyairi and Fishman with human placental microsomes (33).

NADPH dependence of aromatase is shown Fig. 3 (inset). When no NADPH was added, small amounts of estrogens were formed presumably due to small amounts of coenzyme subsisting in the microsomes even after three washings. With 3 μM androstenedione, the apparent K_m was 350 μM for NADPH (Fig. 3). While allowing the release of 3H2O from [1α,2α-3H]androgens, NADPH was nevertheless a much less effective cofactor than NADPH. No aromatization occurred with NADP as cofactor (Fig. 3). NADP 3 mM (NADP/NADPH = 10) produced a 36% inhibition of testosterone aromatization.

Comparison of C-18- and C-19 Androgen Aromatization: Effect of Age

The formation of estrone and estriol from androstenedione, testosterone, norandrostenedione, or 19-nortestosterone was obtained with 100 μM sodium-p-hydroxymercurobenzoate.

**TABLE III**

<table>
<thead>
<tr>
<th>Stereospecific removal of hydrogen atoms at C1 and C2 positions</th>
<th>[1α,2α-3H]-Testosterone</th>
<th>[1α,2α-3H]-Testosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total 3H-androgen activity</td>
<td>612,665</td>
<td>625,312</td>
</tr>
<tr>
<td>3H2O release</td>
<td>24,274</td>
<td>100,774</td>
</tr>
<tr>
<td>3H-estrogen</td>
<td>76,776</td>
<td>59,330</td>
</tr>
<tr>
<td>[3H]Aromatized androgens</td>
<td>101,050</td>
<td>190,104</td>
</tr>
<tr>
<td>3H2O/3H-estrogen</td>
<td>0.32</td>
<td>1.70</td>
</tr>
<tr>
<td>3H in α position of androgens</td>
<td>76%</td>
<td>37% ab</td>
</tr>
<tr>
<td>3H in β position of androgens</td>
<td>24% c</td>
<td>62%</td>
</tr>
</tbody>
</table>

* Obtained from TLC as described under “Experimental Procedures.”

Note: Calculated as 3H-estrogen + 3H2O. Calculated as 3H-estrogen × 100/[3H]Aromatised androgens.

Reed and Ohno (32) found 32% 3H at the α position in the β labeled testosterone from New England Nuclear.

16% indicated by Amersham Corp.

3 μM was measured by RIA (Table IV). Norandrogens were converted to estrogens at least as effectively as were androgens. The highest rate of aromatization was obtained with norandrostenedione as substrate.

17-Hydrox-androgen aromatization resulted mostly in the formation of estradiol and 17-oxo androgen aromatization in estrene formation. 17β-Hydroxytestosterone dehydrogenase contamination led to only 6% interconversion.

An apparent effect of age was observed: aromatase activity was very low at 2 years. It increased 30-fold from 2 to 4 years, then doubled again from 4 to 8 years.

**Inhibition of Androgen Aromatization by Norandrogens**

The inhibitory effect of steroids on 3H2O release from [1α,2α-3H]testosterone or androstenedione is shown in Table V. Aromatization of both androstenedione and testosterone was inhibited with decreasing effect by androstenedione > testosterone > norandrostenedione > 19-nortestosterone > DHT > epitT.

19-Nortestosterone and norandrostenedione were weaker inhibitors than testosterone and androstenedione: the removal of the C-19 methyl group led to the loss of 30% of the inhibitory effect. Norandrostenedione reduced testosterone aromatization more efficiently than 19-nortestosterone reduced androstenedione aromatization, although the inhibition of testosterone and androstenedione aromatization by their corresponding norderivatives was similar.

Androstenedione, with a C-17 ketogroup and a 4-ene-3-oxo configuration exhibited maximal efficiency in inhibiting both androstenedione and testosterone aromatization. The reduction of the 17-ketogroup to a 17β-hydroxyl group led to a 15% loss of inhibitory effect. The formation of the 17α-hydroxy derivative (epiT) resulted in an almost complete loss of inhibitory effect. The 5α-reduction significantly lowered (33–40%) the inhibitory effect.

When using testosterone, 19-nortestosterone, DHT, and epitT at concentrations 7 times that of the substrate, the percentages of androstenedione aromatization inhibition were 75, 32, 24, and 0%, respectively.

**Incubation with C-18 and C-19 Androgens**

In an attempt to determine whether C-18 and C-19 androgens were aromatized by two different pathways, the reaction rate of the combined substrates was compared to that of each individual substrate (34). As shown in Table VI, the reaction velocity of the combined substrates was found to be less than the sum of the reaction velocities measured separately for each of the three sets of substrate pairs. When androstenedione and 19-nortestosterone were incubated together, the amount of estrone formed was 10 times greater than that of estriol.

**DISCUSSION**

Measurement of the release of 3H2O from [1α,2α-3H]androgen is a true reflexion of enzymatic activity (32, 35). The loss of 1α,2α tritium atoms in aromatization is not accompanied by an isotopic effect (32).

The quantities of excreted estrogens in stallion urine are insignificant up to 2 years of age, after which they increase markedly (36). Our results show that aromatase activity in the stallion testis greatly increased after 2 years and then continued to increase up to at least 8 years. This age-related change in the aromatase activity may be linked to the increase in number and volume of the Leydig cells which occurs in the equine testis from ages 2 to 20 (37).

We have shown that as in the human placenta (1, 38), the
Norandrogen Aromatization by Equine Testes Microsomes

TABLE IV

Estrogen formation resulting from testosterone, 19-nortestosterone, androstenedione, and norandrostenedione aromatization

Incubations were performed as described under "Experimental Procedures" with 3 μM androgens or norandrogens. Estrone and estradiol concentrations were measured by RIA. Values (pmol) are the mean (minus the blank) ± S.D. of the results of four assays on seven samples from each incubation. Blank values result from the assay of the same components without incubation. Z values are the sum of estrone + estradiol.

<table>
<thead>
<tr>
<th>Age of stallions</th>
<th>Androstenedione</th>
<th>Norandrostenedione</th>
<th>Testosterone</th>
<th>19-Nortestosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 years</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estrone</td>
<td>157</td>
<td>311</td>
<td>15</td>
<td>18</td>
</tr>
<tr>
<td>Estradiol</td>
<td>11</td>
<td>21</td>
<td>220</td>
<td>251</td>
</tr>
<tr>
<td>Σ</td>
<td>168 ± 28</td>
<td>332 ± 41</td>
<td>235 ± 31</td>
<td>269 ± 7</td>
</tr>
<tr>
<td>8 years</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estrone</td>
<td>355</td>
<td>702</td>
<td>25</td>
<td>34</td>
</tr>
<tr>
<td>Estradiol</td>
<td>17</td>
<td>38</td>
<td>340</td>
<td>571</td>
</tr>
<tr>
<td>Σ</td>
<td>372 ± 73</td>
<td>740 ± 85</td>
<td>365 ± 71</td>
<td>605 ± 4</td>
</tr>
<tr>
<td>Adult</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estrone</td>
<td>725</td>
<td>969</td>
<td>42</td>
<td>38</td>
</tr>
<tr>
<td>Estradiol</td>
<td>9</td>
<td>30</td>
<td>382</td>
<td>438</td>
</tr>
<tr>
<td>Σ</td>
<td>734 ± 113</td>
<td>999 ± 115</td>
<td>424 ± 40</td>
<td>476 ± 68</td>
</tr>
</tbody>
</table>

The aromatization of androgens by placental microsomes involves three sequential hydroxylations and thus requires three molecules of O₂ and NADPH. The first two hydroxylations occur at C-19 (44) and the third at the β position of C-2. The 2β-hydroxy-19-aldehyde derivative formed is highly unstable and collapses non-enzymically (45, 46) to form estrogen by the concerted elimination of the oxidized C-19 residue together with the 1/3 hydrogen atom and the 2β-hydroxy group as formic acid (5, 47). It seems that two catalytic sites are involved, one of which is responsible for the two C-19 hydroxylations and the other for the 2β-hydroxylation (48). On the other hand, placental aromatization of norandrogens (which do not seem to be actual intermediates of C-19 androgen aromatization) involves 1β hydroxylation (7). During aromatization of androgens in normal and diseased human ovaries, stereospecificity of hydrogen loss is also 1β,2β (49) whereas aromatization of androstenedione and norandrostenedione by Pseudomonas testosteroni and Nocardiopsis restrictus seems to involve the loss of 1α-hydrogen (50).

With equine testicular microsomes, 17β-estradiol formed...
Norandrogen Aromatization by Equine Testes Microsomes

**TABLE V**

Effect of different steroids on tritiated testosterone and androstenedione aromatization

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Reaction velocity (pmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Androstenedione</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>Testosterone</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>Norandrostenedione</td>
<td>3.3 ± 0.1</td>
</tr>
<tr>
<td>19-Nortestosterone</td>
<td>5.4 ± 0.1</td>
</tr>
<tr>
<td>DHT</td>
<td>7.7 ± 0.1</td>
</tr>
<tr>
<td>epiT</td>
<td>10.3 ± 0.1</td>
</tr>
</tbody>
</table>

**TABLE VI**

Reaction velocities of aromatase for individual and combined substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reaction velocity (pmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>17.9 ± 1.1</td>
</tr>
<tr>
<td>19-Nortestosterone</td>
<td>20.7 ± 1.7</td>
</tr>
<tr>
<td>Norandrostenedione</td>
<td>19.1 ± 0.6</td>
</tr>
<tr>
<td>Testosterone</td>
<td>17.9 ± 0.6</td>
</tr>
</tbody>
</table>

from [1α,2α-3H]testosterone retained 90% of the tritium whereas 93% of tritium was recovered in H2O released from [1β,2β-3H]testosterone. It might be concluded from the des- tination of the tritium, that in the stallion testis as in the human placenta and ovary, estradiol formation occurs via sterosepecific loss of 1β,2β-hydrogens.

Norandrostenedione and 19-nortestosterone are competitive inhibitors of the aromatization of androstenedione by HPMES (4, 51). Androstenedione and testosterone competitively inhibit the aromatization of each other (32). The magnitudes at 390 and 400 nm of the cytochrome P-450-binding spectra in the presence of androstenedione and 19-nortestosterone are not additive (4). These data suggest that a single enzyme complex is responsible for the aromatization of androstenedione, testosterone, norandrostenedione, and 19-nortestosterone. In granulosa cells, the nonaromatizable DHT is a competitive inhibitor of testosterone aromatization (52). In HPMEs, the aromatizing system for epiT would be different from that for testosterone (53). Competitive inhibitions suggest the existence of a single binding site for androstenedione, testosterone, 19-nortestosterone, norandrostenedione, and DHT. Therefore, the extent of inhibition would be correlated to the respective affinities of the different steroids for this site.

As with HPMES (53), epiT would seem to have no affinity for the ETMES, at least for the active site specific for 17β (or 17-oxo) compound aromatization. The inhibitory effect of the aromatizable steroids showed that the enzyme system had a lower affinity for norandrogens, the decreasing order of apparent affinity being for androstenedione, testosterone, norandrostenedione, and 19-nortestosterone. Androstenedione aromatization with ETMES and HPMES (54) is inhibited to the same extent by testosterone (75 versus 88%) whereas 19-nortestosterone and DHT are much lower inhibitors with ETMES (32 and 24%) than with HPMES (80 and 82%). This raises the possibility that the mechanism of aromatization and the physiological control of the estrogen pool by 3α-reduced C-19 steroids might be somewhat different in the equine testis and human placenta.

With HPMEs, 19-norcompounds are aromatized much more slowly and less effectively than the corresponding methyl compounds (5). Testosterone is converted to estradiol in about 5-fold greater yields than 19-nortestosterone (1, 2), the same yield being obtained with androstenedione and norandrostenedione (3). According to Brody (cited in Ref. 4), "there is at least a 20-fold difference" between C-19-methyl and 19-norandrogen aromatization. Moreover, purified placent al aromatase is far more effective with methylandrogens (6).

With HPMEs, Thomson and Siiteri (55) explain the difference by a less favorable orientation of the norsteroids due to the lack of C-19-methyl group, which would make rate limiting the hydroxylation subsequent to the entry of the second electron into the cytochrome P-450 substrate complex.

In our experiments, norandrogens were aromatized with the same or a greater efficiency than androgens despite their markedly lower affinity for ETMES. This suggests that their less favorable orientation does not result in a rate-limiting step which would reduce their aromatization velocity. However, this might also suggest that the last catalytic intermediate of norandrogen aromatization is less firmly bound to the active site than the 25-hydroxy-19-aldehyde androgen to HPMEs.

The question of whether C-19 and C-18 androgens are aromatized by the same enzyme system remains controversial. Bellino and Osawa (56) propose two sites for androgen aromatization: one site exclusively aromatizing androstenedione, the other aromatizing competitively androstenedione and testoster- one. Inhibition of C-19 and 19-norsteroids by various inhibitors leads Ganguly et al. (7) to conclude that different enzymes could be in operation. On the other hand, based on the fact that 19-nortestosterone is a competitive inhibitor of aromatization and cytochrome P-450 binding of androstenedione, Thomson and Siiteri (55) propose that a single species of cytochrome P-450 is involved in aromatization of 19-nortestosterone and androstenedione. From experiments where androstenedione and testosterone were competitive inhibitors, Reed and Ohno (32) conclude that androstenedione and testosterone are aromatized at a single site.

Our results strongly suggest that the same enzyme system would be involved in testosterone, 19-nortestosterone, norandrostenedione (Table VI), and androstenedione aromatization. The observed rate of aromatization of an equimolar
mixture of two substrates was much lower than the sum of the individual rates, thus clearly indicating the existence of a single aromatization enzyme system in the equine testis, for which androstenedione, testosterone, norandrostenedione, and androstenedione would compete in this decreasing order of affinity. This was confirmed by a 19-nortestosterone aromatization rate which represented about one-tenth of the androstenedione aromatization rate when the two steroids were coincubated in a 1:1 molar ratio.

In conclusion, our investigations suggest that in the equine testis, androgens nor and norandrogens are aromatized by a single enzyme system which would aromatize androgens and norandrogens at the same rate while having a much lower affinity for norandrogens.

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