OCCURRENCE OF 19-OXOANDROST-4-ENE-3,17-DIONE IN THE COURSE OF OESTROGEN BIOSYNTHESIS BY EQUINE TESTICULAR MICROSOMES

By
Ran Oh and Bun-ichi Tamaoki

ABSTRACT

Radioactive 19-oxoandrost-4-ene-3,17-dione was obtained as one of the metabolites produced from the $^{14}$C-labelled androstenedione and 19-hydroxy-androst-4-ene-3,17-dione by equine testicular microsomes (10 000–105 000 × g precipitate) under an aerobic condition. Under an anaerobic condition, however, the production of 19-oxoandrostenedione and oestrone from the above two precursors was severely reduced. Identification of 19-oxoandrostenedione was based on the following criteria: 1. Mobilities of the metabolite were identical with those of authentic preparation of 19-oxoandrostenedione on thin layer chromatograms developed in several different solvent systems. 2. Other physico-chemical characteristics of the metabolite such as ultraviolet absorption spectrum, and behaviour against chemical reagents were similar to those of authentic 19-oxoandrostenedione. 3. Specific activities of the crystal and the solid in mother liquor were found to be constant, when the $^{14}$C-labelled metabolite was repeatedly crystallized with authentic 19-oxoandrostenedione. 4. Moreover, when the $^{14}$C-labelled metabolite was recrystallized with authentic $^3$H-labelled 19-oxoandrostenedione, ratios of the two radioisotopes of the crystal and the solid in mother liquor were constant. The time course of the metabolism of androstenedione and 19-hydroxyandrostenedione revealed that the metabolic fate of 19-oxoandrostenedione had characteristics compatible with an intermediate in the oestrone synthesis from the above two precursors. The role of this particular metabolite was discussed in relation to the oestrogen synthesis in the equine testicular tissue.

In the previous paper (Oh & Tamaoki 1970), we reported on the in vitro oestrogen biosynthesis by the microsomal fraction of equine testicular tissue. When androstenedione and 19-hydroxyandrostenedione were incubated separately...
with the equine testicular microsomal fraction under an aerobic condition, one metabolite which had not yet been identified was found among the metabolites. This paper deals with the occurrence and identification of this metabolite and its role in relation to oestrogen biosynthesis in equine testicular tissue.

**Materials and Methods**

*Trivial names and abbreviations*


*Tissue preparation*

Immediately after orchietomy of horses, the testicular glands were isolated from the connective tissue, chilled on ice and then transferred to the laboratory. After decapsulation, the testicular tissue was homogenized first with a Waring blender and then with a loose-fitting Teflon-glass homogenizer. After the nuclear and mitochondrial fractions had been removed from the homogenates, the microsomal fraction was obtained as the precipitate at 105,000 × g as previously reported (Oh & Tamaoki 1970), and was used as the enzyme preparation for the investigation of the steroidogenesis.

*Steroids and other chemicals*

The authentic preparation of 19-oxoandrostenedione was kindly supplied by Dr. L. R. Axelrod, and [16-3H]19-oxodehydroepiandrosterone (0.15 mCi/mmole) was a gift of Dr. S. J. M. Skinner. 19-Hydroxyandrostenedione was kindly offered by Mr. W. H. Rooks II. 14C-labelled 19-hydroxyandrostenedione was used as a substrate, after it had been firmly identified as previously reported (Oh & Tamaoki 1970). 4-14C-labelled dehydroepiandrosterone (42 mCi/mmole) and androstenedione (35 mCi/mmole) and 1,2-3H-labelled androstenedione (50 Ci/mmole) were purchased from New England Nuclear Corp., Boston, Mass. Their radiochemical purities were confirmed by thin layer chromatography on silica gel before use. Each steroid was diluted so as to have an appropriate specific activity with the respective non-radioactive steroid preparation which was commercially obtained. NADPH and NAD were purchased from Boehringer and Soehne, Mannheim, Germany. All solvents used in this experiment were redistilled before use.

*Incubation and separation of the metabolites*

The incubation mixture was generally prepared so as to consist of equine testicular
microsomal fraction (1–5.2 mg protein), the substrate (30–350 nmole, 4–10 \times 10^4 \text{ cpm}) and NADPH (final concentration, 240 \mu\text{m}) in a 0.25 M sucrose solution buffered at pH 7.4 with Tris (final concentration, 10 mM) and HCl. The final volume of the mixture in a flask was adjusted to 5 ml, and incubation was carried out at 37°C for 60 min in an atmosphere of a mixture of O\textsubscript{2} and CO\textsubscript{2} (95:5), unless otherwise mentioned. More details related to the incubation have been individually stated together with the results obtained.

Immediately after the incubation, 15 ml of methylenedichloride was added to the incubation flask, and the mixture was vigorously shaken to terminate enzyme reactions. The methylenedichloride layer was collected and dried with anhydrous Na\textsubscript{2}SO\textsubscript{4}. After adding authentic progesterone, androstenedione, 17\alpha-hydroxyprogesterone, testosterone and 11-deoxycortisol as carrier or marker steroids to the extract, this was subjected to thin layer chromatographic analysis on silica gel or alumina in several solvent systems. Developed plates were examined under ultraviolet light (254 nm) to detect spots of the carrier \textsuperscript{14}C-\textsuperscript{3}H steroids. To find radioactive spots on the chromatograms, the plates were autoradiographically examined by exposing a sheet of X-ray film to the plates for about 7 days, and also examined by a windowless flow-gas counter with a scanning device (Vangurard, 880 & 885 T. M. C., North Haven, Conn.). Spots of the metabolites thus detected were scraped off from the plates, and the steroids were extracted with a solvent mixture of chloroform and ethanol (1:1 by vol.).

**Determination of radioactivity**

Radioactivities due to \textsuperscript{14}C and \textsuperscript{3}H in the steroid metabolites were quantitatively measured by a liquid scintillation spectrometer (725 System, Nuclear-Chicago, Des Plaines, Ill.), after the extracts had been dissolved in 11 ml of the toluene solution which contained 0.4 \% 2,5-diphenyloxazole (PPO) and 0.01 \% 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP). The counting efficiency of radiocarbon was about 71 \% with the background 18 cpm, and in the case of double tracer counting, 57 \% with 3 cpm background for \textsuperscript{14}C and 32 \% with 2 cpm for tritium.

**RESULTS**

**Metabolism of androstenedione by the equine testicular microsome**

Fig. 1 shows the distribution of the radioactivities due to the metabolites of the radioactive androstenedione on a thin layer chromatogram. In addition to the metabolites which had been identified as previously reported (Oh & Tamaoki 1970), there was a metabolite mentioned as X in Fig. 1 which showed a mobility a little more polar than the androstenedione (substrate) on the chromatogram. This metabolite will be hereafter designated as Metabolite X. Though it will be stated later in the time course study, Metabolite X was obtained as a metabolic product of either androstenedione or 19-hydroxy-androstenedione under an aerobic condition.

**Physico-chemical characteristics of Metabolite X**

The physico-chemical features of Metabolite X were listed as follows:

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Radioscannogram of the metabolites of [4-14C]androstenedione with equine testicular microsomes.
[4-14C]Androstenedione (350 nmole, 10 × 10⁴ cpm) was incubated with equine testicular microsomal fraction (1 mg protein) in the presence of NADPH under an aerobic condition for 20 min. OE₁, A, T, 19-OH A and X denote respectively oestrone, androstenedione, testosterone, 19-hydroxyandrostenedione and Metabolite X.

1. Metabolite X did not have any hydroxy group(s) which was acetylatable with acetic anhydride and pyridine at room temperature overnight.

2. Metabolite X was not a further product of oestrone, since no radioactive metabolite corresponding to Metabolite X was detectable, when radioactive oestrone was incubated with the equine testicular microsomes.

3. After incubation of a mixture of 1,2-3H- and ¹⁴C-labelled androstenedione with the microsomal fraction of equine testes, no significant loss of the tritium relative to radiocarbon was observed in the fraction of Metabolite X, whereas the ratio in the oestrone fraction was reduced to less than half of the one found in the C-19 steroids (Table 1). This result indicated that Metabolite X was not a 1,2-dehydrated metabolite.

4. The above mentioned results was supported by the following ultraviolet absorption spectrum of Metabolite X: λ<sub>Maximum</sub> = 240 nm, and its molecular extinction coefficient was 18 000 as C₁₉H₂₄O₃.

5. Finally, Metabolite X was chromatographically found to be identical with the oxidation product of 19-hydroxyandrostenedione with pyridine-CrO₃ complex (Skinner & Akhtar 1969).
Table 1.
Metabolism of a mixture of [4-14C] androstenedione and [1,2-3H] androstenedione by equine testicular microsomes.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>3H (cpm)</th>
<th>14C (cpm)</th>
<th>3H/14C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Androstenedione (Substrate)</td>
<td>326 900</td>
<td>16 600</td>
<td>19.7</td>
</tr>
<tr>
<td>Testosterone</td>
<td>52 600</td>
<td>29 000</td>
<td>18.1</td>
</tr>
<tr>
<td>19-Hydroxyandrostenedione</td>
<td>60 500</td>
<td>31 000</td>
<td>19.3</td>
</tr>
<tr>
<td>Metabolite X</td>
<td>15 500</td>
<td>80 000</td>
<td>19.4</td>
</tr>
<tr>
<td>Oestrone</td>
<td>17 800</td>
<td>21 000</td>
<td>8.5</td>
</tr>
</tbody>
</table>

The mixture of [1,2-3H] (70 × 10^4 cpm)- and [4-14C] (5 × 10^4 cpm)- androstenedione (total amount 175 nmole) was incubated with the equine testicular microsomal fraction (1 mg protein).

From the above mentioned characteristics of Metabolite X, it was considered as 19-oxoandrostenedione and subjected to identification.

Identification of Metabolite X as 19-oxoandrostenedione

The structure of Metabolite X was elucidated by the following procedures.
1. Mobilities of Metabolite X were identical with those of the authentic 19-oxoandrostenedione on silica gel- and alumina-coated thin layer chromatograms developed in several different solvent systems. \( R_F \) values of the authentic 19-oxoandrostenedione are listed in Table 2 together with those of the related

Table 2.
\( R_F \) values of the authentic 19-oxoandrostenedione and other related steroids on thin layer chromatogram.

<table>
<thead>
<tr>
<th>Absorbent</th>
<th>Solvent system</th>
<th>( R_F ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( A )</td>
<td>19-Oxo</td>
</tr>
<tr>
<td>Silica gel*</td>
<td>Benzene: Acetone (4:1)</td>
<td>0.72</td>
</tr>
<tr>
<td>Alumina**</td>
<td>Benzene: Acetone (3:2)</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>Benzene: Acetone (1:1)</td>
<td>0.75</td>
</tr>
</tbody>
</table>

* Silica gel G and GF (4:1 by weight) (Merck).
** Alumina G and GF (4:1 by weight) (Merck).
steroids. When the radioactive Metabolite X and the authentic preparation of 19-oxoandrostenedione were simultaneously analyzed on the same thin layer plates, the mobilities of the radioactive spots were confirmed to be identical with the ultraviolet-light absorbing spots of the authentic preparation.

2. The chemical behaviour of Metabolite X was similar to that of the authentic 19-oxoandrostenedione preparation, such as resistance against acetylation, oxidation and pipsylation (Oh & Tamaoki 1970).

3. The radioactive Metabolite X which was derived from [4-14C]androstenedione by the equine testicular microsomes was further purified by repeated thin layer chromatography, and then crystallized with the authentic preparation of 19-oxoandrostenedione in different solvent systems. As shown in Table 3, specific activities of the crystal and the solid in mother liquor obtained in the course of crystallization was found to be constant within the experimental errors.

4. Tritiated 19-oxoandrostenedione was enzymatically prepared from the authentic [16-3H] 19-oxodehydroepiandrosterone under anaerobic condition in the presence of NAD by the testicular microsomal fraction of rats which was reported to contain A5-3β-hydroxysteroid dehydrogenase and A5-A4 isomerase (Inano et al. 1970). The tritiated product which was found on the spot of 19-oxoandrostenedione added as carrier was identified with the above mentioned authentic preparation as 19-oxoandrostenedione by further thin layer chromatography. After crystallization of the 14C-labelled Metabolite X with the above [3H] 19-oxoandrostenedione, ratios of the two radionuclides in the crystal and the solid in mother liquor became constant after a few crystallizations, as shown in Table 4.

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>Specific activities (CPM/mg)*</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>solid in mother liquor</td>
<td>crystal</td>
</tr>
<tr>
<td>Methylene dichloride</td>
<td>n-heptane</td>
<td>380</td>
</tr>
<tr>
<td>Acetone</td>
<td>n-heptane</td>
<td>239</td>
</tr>
<tr>
<td>Benzene</td>
<td>n-heptane</td>
<td>292</td>
</tr>
<tr>
<td>Dioxane</td>
<td>n-heptane</td>
<td>232</td>
</tr>
<tr>
<td>Tetrahydrofuran</td>
<td>n-heptane</td>
<td>235</td>
</tr>
</tbody>
</table>

* calculated specific activity: 272 CPM/mg.
Occurrence of 19-oxoandrostenedione in the time course of metabolism of androstenedione and 19-hydroxyandrostenedione

When androstenedione and 19-hydroxyandrostenedione were separately incubated with the equine testicular microsomes in the presence of NADPH and under an aerobic condition, amounts of the substrates and the metabolic products were measured successively at fixed or regular intervals. The results are illustrated as Figs. 2 and 3. The substrate or androstenedione was consumed almost exponentially, while the production of oestrone quickly increased and reached a plateau 5-10 min after the start of the incubation, as shown in Fig. 2. Meanwhile, 19-hydroxyandrostenedione and 19-oxoandrostenedione were gradually produced up to certain times of incubation (2 and 3 min respectively), and thereafter, both metabolites started to decrease, showing peaks against the elapsed time of incubation. The peak of the 19-hydroxyandrostenedione production was followed by the peak of the 19-oxoandrostenedione production.

As shown in Fig. 3, the exponential consumption of the substrate or 19-hydroxyandrostenedione was observed with the elapsed time of incubation and oestrone was produced increasingly up to the end of the incubation. Meanwhile, 19-oxoandrostenedione was also produced along the time of incubation, reaching a maximum 35 min after incubation, after which it started to decrease.

Simultaneous occurrence of 19-oxoandrostenedione with the oestrone produced from androgens

When androstenedione and 19-hydroxyandrostenedione were separately incubated with the equine testicular microsomes in the presence of NADPH and under an anaerobic condition, neither oestrone nor 19-oxoandrostenedione were...
Fig. 2.

Time course of transformation of androstenedione with the equine testicular microsomal fraction.
[4-14C]Androstenedione (35 nmol, $10 \times 10^4$ cpm) was incubated with the equine testicular microsomal fraction (2 mg protein) in the presence of NADPH under an aerobic condition.

practically detectable as a metabolite of the substrates (Table 5). When 19-hydroxyandrostenedione was incubated in the presence of NAD as a hydrogen acceptor under an anaerobic condition, the production of 19-oxoandrostenedione and also of oestrone from the substrate was markedly reduced, in comparison with those obtained with NADPH under an aerobic condition.

**DISCUSSION**

In view of the fact that the production of 19-oxoandrostenedione from 19-hydroxyandrostenedione was remarkably reduced under an anaerobic condition, it is concluded that this enzymatic reaction seemed entirely different from the one of the usual NAD(P)-dependent hydroxysteroid oxido-reductase which does not require molecular oxygen. This result was in agreement with the previous work reported by Skinner & Akhtar (1969) in that, in the course of biotransformation of 19-hydroxyandrostenedione to 19-oxoandrostenedione, NADPH and molecular oxygen were required by human placental microsomes, thus suggesting 19-dihydroxyandrostenedione as the intermediate. As shown in
Fig. 3.
Time course of the metabolism of 19-hydroxyandrostenedione with the equine testicular microsomal fraction.

[4-14C]19-hydroxyandrostenedione (132 nmole, $4 \times 10^4$ cpm) was incubated with the equine testicular microsomal fraction (1 mg protein) in the presence of NADPH under an aerobic condition.

Table 5, a significant increase in testosterone production from androstenedione was noted under the anaerobic condition, in contrast to the drastic decrease in the production of 19-oxoandrostenedione and oestrone. This suggests that the 19-hydroxylase and 17β-hydroxysteroid dehydrogenase in the equine testicular microsome shared androstenedione as the common substrate and consumed it competitively.

When androstenedione and 19-hydroxyandrostenedione were supplied as substrates in amounts sufficient to saturate the same enzyme system, a significant amount of 19-oxoandrostenedione was detected, simultaneously with the appearance of oestrone. On the contrary, when progesterone and 17α-hydroxyprogesterone were used as substrates for the equine testicular microsomes, no detectable amount of 19-oxoandrostenedione accumulated in the medium (Oh & Tamaoki 1970).

There are limited number of reports (Axelrod & Goldzieher 1962; Akhtar & Skinner 1968) on the definite identification of 19-oxoandrostenedione biotransformed by oestrogen-producing tissue, probably because of the minute accumulation of this metabolite in the medium, chromatographically close polarity to androstenedione, and its chemically labile nature. In fact, the actual amount of 19-oxoandrostenedione produced during the incubation was limited,
Table 5.
Influence of incubation atmosphere and cofactors on the metabolism of androstenedione (Experiment I) and 19-hydroxyandrostenedione (Experiment II) with equine testicular microsomes.

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>NADPH</th>
<th>NAD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O₂</td>
<td>Ar</td>
</tr>
<tr>
<td>Gas phase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Androstenedione (substrate)</td>
<td>1.3</td>
<td>13.0</td>
</tr>
<tr>
<td>19-Hydroxyandrostenedione</td>
<td>1.3</td>
<td>6.3</td>
</tr>
<tr>
<td>Testosterone</td>
<td>1.3</td>
<td>13.3</td>
</tr>
<tr>
<td>Oestrone</td>
<td>28.8</td>
<td>0.3</td>
</tr>
<tr>
<td>19-Oxoandrostenedione</td>
<td>0.2</td>
<td>0.0</td>
</tr>
<tr>
<td>Experiment II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19-Hydroxyandrostenedione (substrate)</td>
<td>6.7</td>
<td>26.9</td>
</tr>
<tr>
<td>Oestrone</td>
<td>19.9</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Figures in Table 5 denote the amount of the steroids in nmole.
[4-¹⁴C]Androstenedione (35 nmole, 10 × 10⁴ cpm) and [4-¹⁴C]19-hydroxyandrostenedione (35 nmole, 10 × 10⁴ cpm) were incubated with the equine testicular microsomal fraction (5.2 mg protein) in the presence of NADPH or NAD (final concentration respectively 240 μM and 301 μM).

but with sufficient radioactivity in our preliminary experiments. During the purification procedure of this metabolite, we found that when a minute amount of this metabolite was analyzed on a silica gel thin layer chromatogram, it was easily transformed into an artifact which no longer showed characteristic absorption around 240 nm and its mobility on the thin layer chromatogram became less polar than its initial one. 19-oxotestosterone-17β-acetate was susceptible to ultraviolet irradiation at room temperature (Pfenninger et al. 1968), though it is not certain whether or not this type of transformation is directly related to the presently discussed denaturation of 19-oxoandrostenedione. To protect the metabolite from this unexpected alteration, we added the carrier steroid of 19-oxoandrostenedione to the steroid extract before the chromatographic analysis.

As one of the obligatory steps for aromatization by human placental aromatase, stereospecific removal of the hydrogen atoms at C-1β and C-2β was recently reported (Brodie et al. 1969; Fishman et al. 1969; Townsley & Brodie 1968). From the present experimental results using as the substrate, 1,2-tritiated
androstenedione (distribution of tritium C-1 vs C-2 was 1:1, and distribution of tritium at C-1 was 75% in β, and 25% in α, but the quantitative distribution at C-2 was not known (Morato et al. 1962). It was suggested that the same type of the C-1 and C-2 dehydrogenation process would be involved in the course of aromatization with equine testicular tissue, after 19-hydroxylation of androstenedione, and then probably after oxidation of its 19-hydroxy group.

On the role of 19-oxoandrostenedione in the course of oestrone biosynthesis, the following should be considered:

1. 19-Oxoandrostenedione is a metabolite of 19-hydroxyandrostenedione which is regarded as an obligatory intermediate in the course of oestrone biosynthesis from androstenedione (Meyer 1955; Longchampt et al. 1960).

2. The angular methyl group at 19-position of androgens is removed in the form of formaldehyde in the course of aromatization with placental enzyme system (Breuer & Grill 1961; Axelrod et al. 1965).

3. 19-Oxoandrostenedione had been reported as being more efficiently converted to oestrone than 19-hydroxyandrostenedione by human placental microsomes (Morato et al. 1961) and is also transformed to oestrone by the equine testicular microsomes (unpublished data).

4. 19-Oxoandrostenedione shows a characteristic fate compatible with an intermediate in the time course of oestrogen formation, being similar to the fate of 19-hydroxyandrostenedione (Wilcox & Engel 1965).

5. Moreover, a metabolic sequence from 19-oxoandrostenedione to oestrone has been suggested from their simultaneous occurrence and disappearance due to the different atmospheric phases (Table 5).

From the above mentioned results, the following pathway is proposed for the aromatization by the equine testicular microsomes: androstenedione → 19-hydroxyandrostenedione → 19-oxoandrostenedione → oestrone, even though the production of oestrone directly from 19-hydroxyandrostenedione without any dehydrogenation of C-19 moiety could not be excluded.

The mechanism and pathways related to the equine testicular aromatization of androgens are being further investigated in this laboratory.

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REFERENCES


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