Enzymatic Aromatization of Steroids

I. EFFECTS OF OXYGEN AND CARBON MONOXIDE ON THE INTERMEDIATE STEPS OF ESTROGEN BIOSYNTHESIS*

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SUMMARY

Intermediate steps in the aromatization of 4-androstene-3,17-dione to estrone, catalyzed by human placental microsomes, have been evaluated through the use of normal and hypothetical intermediate compounds as substrates and the oxidases involved have been characterized by the effects of oxygen and carbon monoxide upon these conversions. When air was replaced by a 5% oxygen-95% nitrogen atmosphere, the rate of aromatization of 4-androstene-3,17-dione or of 19-hydroxy-4-androstene-3,17-dione was reduced 30 to 35% while no inhibition occurred if 3,17-dioxo-4-androsten-19-ol was the substrate. The aromatization of all three of these steroids in 5% oxygen was not inhibited when carbon monoxide was added at a concentration 8.8 times that of oxygen. However, this same CO:O2 ratio of 8.8 decreased the aromatization of 4-estrene-3,17-dione 51 to 95% and the aromatization of 1,4-androstadiene-3,17-dione 10 to 16%. Hydroxylation of the A ring of 4-estrene-3,17-dione was also inhibited by carbon monoxide to the same extent as was its aromatization. These findings have been interpreted as evidence for the existence of three mixed function oxidases active in steroid aromatization, one of which may be a form of cytochrome P-450.

The aromatization of androstenedione1 to estrone, catalyzed by human placental microsomes is a complex conversion involving both demethylation and desaturation. Requirements for both O2 and TPNH (1) suggest the participation of an enzyme of the mixed function oxidase type (2). Demonstrations of 19-oxygenated intermediates, 19-hydroxyandrostenedione (2–5), and 19-oxoandrostenedione (6, 7), indicate a multistep reaction sequence (Scheme 1). But findings of identical requirements for O2 and TPNH for the aromatization of all intermediates (6, 7), provide no discrimination as to the number of oxidases participating in the over-all conversion.

We have previously reported that cytochrome P-450, a component of many mixed function oxidase systems utilizing steroid substrates (8–13), is present in placental microsomes but appears not to function as the oxidase component in the rate-limiting step of aromatization since CO failed to inhibit the over-all conversion of androstenedione to estrone under conditions of limiting O2 supply (14). We have now extended this study to examine each known intermediate step in the aromatization sequence, utilizing O2 and CO atmospheres to expose the properties of the participating oxidase.

A preliminary report of some of our findings has already appeared (15).

EXPERIMENTAL PROCEDURES

Solvents used for routine extractions and chromatography were reagent grade; those used for purification, crystallization, or solution of steroid substrates were redistilled or were of spectroscopic quality. TPNH was prepared by chemical reduction of TPK+ (P-L Biochemicals) with the method of Kaplan, Colowick, and Neufeld (16). Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) was obtained from Boehringer Mannheim. Gas mixtures were prepared and analyzed by The Matheson Company, Inc., East Rutherford, New Jersey.

Steroid Substrates—Androstenedione and 1,4-androstadiene-dione were purchased from Sigma. The latter was recrystallized four times from benzene and once from cyclohexane. Substrate solutions of the purified material were prepared just prior to each incubation.

4-14C-19-Norandrostenedione was prepared by allowing a 1 mM solution of 4-14C-17β-hydroxy-4-estren-3-one (Amersham-Searle, Des Plaines, Illinois; specific activity 50.2 mCi per nmole) in glacial acetic acid to react with a molar excess of CrO3 for 5 min at room temperature. The 4-14C-19-norandrostenedione formed was purified by paper chromatography in System I and was diluted with unlabeled 19-norandrostenedione (Dextran Chemicals, New York, New York) to a specific activity of 0.654 mCi per nmole.

19-Oxandrostenedione was prepared from 19-hydroxyan-

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1 The non systematic names of steroids used are: androstenedione, 4-androstene-3,17-dione; 19-hydroxyandrostenedione, 19-hydroxy-4-androstene-3,17-dione; 19-oxoandrostenedione, 3,17-dioxo-4-androsten-19-ol; 1,4-androstadienedione, 1,4-androsta-
diene-3,17-dione; 19-norandrostenedione, 4-estrene-3,17-dione.
Engel et al. (21), with estrone as standard. Since estrone and 17β-estradiol react with unequal intensity in this procedure, any experimental results, measured in terms of total estrogens, which could have arisen merely by a shift in estrone-17β-estradiol interconversion were subjected to further analysis of the individual estrogens resolved by paper chromatography in System II. Estrone or 17β-estradiol were then used as standards for the appropriate estrogen fractions.

In addition, fluorometric findings were confirmed semi-quantitatively by chromatography of steroid fractions on thin layers of Silica Gel G in diethyl ether-benzene (2:1). Estrogens were detected and characterized by their reactivity with both the FeCl₃-K₃Fe(CN)₆ (23) and H₂SO₄-ethanol (5) reagents.

Processing of 4,19-Norandrostenedione Incubation Mixtures and Characterization of Products—Chloroform extracts were purified with the hexane-90% methanol partition step and then fractionated by paper chromatography in System II. No estrone (RF 0.74) was found but 17β-estradiol (RF 0.38) was detected and was retained in the phenolic fraction on further purification. After quantitative assay it was rechromatographed in System II and shown to have the same mobility as an authentic sample as determined both by detection of radioactivity and reactivity with the FeCl₃-K₃Fe(CN)₆ reagent. No contaminants could be detected by either method.

A second, polar, metabolite (RF 0.23) of 19-norandrostenedione was also detected in the initial chromatogram in System II. It had the mobility expected for the 1β- or 2β-hydroxylated products indicated by Townsley and Brodie (24) and Brodie, Pillai, and Hay (25). It had a reactivity in the acid fluorescence assay comparable to estrone and on treatment with 1% NaOH for 3 hours at room temperature it yielded a product which displayed a mobility identical with that of estrone (RF 0.74, System II; $E_{210}$ 0.51, System I) when detected by reactivity with FeCl₃-K₃Fe(CN)₆ and by localization of radioactivity.

The A ring-hydroxylated metabolite and estradiol were formed in a ratio of 1:7 (cf Reference 24).

Radioactivity was detected on paper chromatograms with a Packard model 7201 scanner and was measured quantitatively by liquid scintillation spectrometry as previously described (14).

Paper Chromatography—Chromatographic development was carried out at room temperature. Solvent systems contained ligroin-toluene-methanol-water in the following proportions by volume: System I, 9:1:8.5:1.5; System II, 5:5:8.5:1.5. Whatman No. 1 filter paper, washed with 2 N acetic acid, water, and methanol as recommended by Axelrod (26), was used whenever steroids were later to be eluted. Absolute methanol was the eluting solvent.

RESULTS

Effects of Oxygen—Data from several laboratories (4–7, 18, 27, 28) indicate that when the aromatization reaction is carried out in air, the rate-limiting step is the formation of the 19-hydroxylated, obligatory intermediate, and that the aromatization of the 19-oxo intermediate proceeds as fast or faster than the conversion of the 19-hydroxy compound. Since our
TABLE I

Effect of oxygen on aromatization

Assay conditions are described under "Experimental Procedures." Relative rates of reaction are based upon a value of 100 assigned to the rate of aromatization of androstenedione in air. Absolute rates for this conversion ranged from 100 to 196 pmoles per min per mg of protein. Each value is the average of at least two experiments with different batches of microsomes. Each experiment was performed in duplicate.

<table>
<thead>
<tr>
<th>Atmosphere</th>
<th>From androstenedione</th>
<th>From 19-hydroxyandrostenedione</th>
<th>From 19-oxoandrostenedione</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>100</td>
<td>208</td>
<td>702</td>
</tr>
<tr>
<td>5% O₂-95% N₂</td>
<td>70</td>
<td>140</td>
<td>702</td>
</tr>
<tr>
<td>100% N₂</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table I also reveals that the aromatization of both androstenedione and 19-hydroxyandrostenedione is diminished to approximately the same extent when air is replaced by 5% O₂. Results of these experiments are pre-
sented in Table I. In air, the expected pattern of relative activities was observed. However, the effectiveness of the 19-oxo compound as a substrate for aromatization was more striking than had been indicated by previous studies which had not measured initial reaction rates (6, 7, 18). In 5% O₂, as well, a progressive increase in effectiveness as substrate followed the sequential oxidations at C-19 of the C₁₉ steroids. Therefore, within this range of O₂ concentrations, the rate-limiting step measured in the aromatization of androstenedione will be its hydroxylation at C-19 (Step I, Scheme 1) and the rates of aromatization of 19-hydroxyandrostenedione and of 19-oxoandrostenedione will each measure a different and distinct intermediary step of the aromatization process (Step II, a or b and Step III, respectively).

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<table>
<thead>
<tr>
<th>Atmosphere</th>
<th>Relative rates of estroline formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>From androstenedione</td>
</tr>
<tr>
<td>------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>Air</td>
<td>100</td>
</tr>
<tr>
<td>5% O₂-95% N₂</td>
<td>70</td>
</tr>
<tr>
<td>100% N₂</td>
<td>0</td>
</tr>
</tbody>
</table>

TABLE II

Effect of carbon monoxide on aromatization

Assay conditions are described under "Experimental Procedures." Each substrate was tested with at least two different batches of microsomes. Each experiment included duplicate pairs of flasks, with and without CO. Values listed are the ranges observed in the various experiments.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>In 5% O₂-95% N₂</th>
<th>In 5% CO-95% N₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmoles/min/mg protein</td>
<td>% activity in 5% O₂-95% N₂</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>38-62</td>
<td>8-26</td>
</tr>
<tr>
<td>19-Hydroxyandrostenedione</td>
<td>157-192</td>
<td>107-192</td>
</tr>
<tr>
<td>19-Oxandrostenedione</td>
<td>908-1,060</td>
<td>968-1,060</td>
</tr>
<tr>
<td>1,4-Androstadienedione</td>
<td>38-62</td>
<td>38-62</td>
</tr>
<tr>
<td>19-Norandrostenedione</td>
<td>9-20</td>
<td>9-20</td>
</tr>
</tbody>
</table>

Since the aromatization of all three of these compounds still occurs when the O₂ concentration is reduced to 1%, aromatization of 19-norandrostenedione, which involves only dehydrogenation, is markedly inhibited by CO.

Townley and Brodie (24) and Brodie et al. (25) showed that 19-norandrostenedione can undergo enzymatic hydroxylation in ring A under the incubation conditions of the aromatization assay and they have pointed out that these hydroxylated products can also give rise to aromatic steroids by acid or base catalyzed dehydrations. Such a nonenzymatic generation of estrone could occur during the fluorescence assay of unfractiated extracts or during the alkaline partition used for purification of the reaction products. Therefore, additional incubations were carried out with 4-¹⁴C-19-norandrostenedione as substrate. Hydroxylated and aromatic products were fractionated by paper chromatography to prevent nonenzymatic aromatization and results from the fluorescence assay were checked by measurements of radioactivity. In two experiments, each with duplicate pairs of flasks, CO was found to inhibit the enzymatic aromatization of 19-norandrostenedione 88 to 94% while the conversion to the hydroxylated metabolite or metabolites was also reduced to the same extent.

DISCUSSION

Current knowledge concerning the intermediate steps of estrogen biosynthesis from C₁₉ steroid precursors is summarized by Scheme 1. Hydroxylation at C-19 is the first, and an obligatory step in the aromatization of androstenedione (3-5). 19-Oxandrostenedione can also serve as an intermediate (6, 7), but a mandatory role has yet to be established and alternative pathways (Step II, a and b) are therefore depicted for the conversion of the 19-hydroxylated compound to estrone.

Under usual experimental conditions, these intermediates do not accumulate, since each step can occur more rapidly than the one preceding it in the sequence. This has permitted our use of intermediates as substrates to measure rates of discrete segments of the complete aromatization reaction.

Each intermediate step, so far distinguished, shares the same requirements for O₂ and TPNH characteristic of the over-all conversion but could potentially be catalyzed by a unique mixed
function oxidase. We have investigated the effects of \( \text{O}_2 \) and CO on each of these separate steps in order to define the enzymatic properties of the oxidase system involved.

Our studies with CO provide no differentiation of oxidase activities at Steps I, II, or III. Aromatization of androstenedione, 19-hydroxyandrostenedione, and 19-oxoandrostenedione are all insensitive to this reagent under our experimental conditions. There is therefore, no evidence for the participation of cytochrome P-450 at any stage of the aromatization sequence. The lack of inhibition by CO even when \( \text{O}_2 \) supply is rate limiting would preclude cytochrome P-450 as a participant in the oxidations occurring at C-19 during Steps I and II.

Gaylord and Mason (29) have also found that cytochrome P-450 does not serve as an oxidase in the oxidative demethylation of 4a-methylsterols, a multi-step process which also is initiated by an aerobic hydroxylation of a methyl group attached to a steroid ring.

A difference in oxidase properties is discernable, however, in incubations carried out under reduced \( \text{O}_2 \) tension. Aromatization of the 19-oxo intermediate is selectively insensitive to low \( \text{O}_2 \) levels. Thus the oxidase acting at Step III appears to be characterized by a higher affinity for \( \text{O}_2 \) and can be differentiated from that promoting either Steps I or II.

No discrimination between oxidase activities at Steps I and II has been possible, however, since the aromatization of androstenedione and 19-hydroxyandrostenedione are both inhibited to the same degree when \( \text{O}_2 \) supply is restricted. Akhtar and Skinner (6) have postulated that Step II may actually be a second hydroxylation of C-19. If this is true, a single 19-hydroxylation might well promote both Steps I and IIa.

The attempt to resolve artificially the demethylation and dehydrogenation components of the aromatization process through the use of 1,4-androstadiene-3,17-dione and 19-norandrostenedione as model substrates has revealed effects of CO not encountered when the aromatization of the normal intermediates was examined.

Thus a third oxidase of placental microsomes involved in the aromatization of 19-norandrostenedione can be defined by its CO sensitivity. By use of Warburg's partition equation (30), partition constants ranging from 0.56 to 1.2 were calculated for this oxidase from the extent of inhibition obtained when 4\( ^4 \)\( \text{C} \)-19-norandrostenedione was used as substrate and products were extensively purified. These results are in good agreement with values obtained by others for a number of cytochrome P-450-catalyzed steroid oxidations (8-13).

Since the aromatization of 19-norandrostenedione involves a stereospecific loss of the same \( \text{H} \) hydrogen eliminated during this oxidase from the extent of inhibition obtained when 4\( ^4 \)\( \text{C} \) partition constants ranging from 0.56 to 1.2 were calculated for values obtained by others for a number of cytochrome P-450 as model substrates has revealed effects of CO not encountered when the aromatization of the normal intermediates was examined.

Alternatively, the aromatization of C\( 18 \) and C\( 19 \) steroids may be distinct metabolic processes and may share no common mechanism or catalyst. In this regard, it is interesting that the unique formation of the A ring hydroxylated metabolite or metabolites (24, 25) of 19-norandrostenedione is also inhibited by CO and to the same extent as is its aromatization.

The suitability of 1,4-androstadiene-3,17-dione as a model substrate for C-19 demethylation is even less secure. It is not known whether, as with other C\( 19 \) steroids, 19-hydroxylation is an obligatory or rate-limiting feature of its conversion. Nor, if 19-oxygenated intermediates are formed, is it known whether enzymatic or nonenzymatic (33-35) processes are responsible for their subsequent aromatization. The limited inhibition by CO observed with this substrate, therefore, can not be interpreted decisively. It could result from (a) the inhibition of a rate-limiting oxidase having a low affinity for carbon monoxide, (b) the inhibition of a CO-sensitive but nonrate-limiting step in a reaction sequence to the point where it did become rate limiting, or (c) the summation of effects on a major CO-insensitive pathway and a parallel or competing, minor, CO-sensitive pathway.

We have interpreted our experimental results as giving evidence for the existence of multiple oxidases participating in steroid aromatization. Physical fractionation of these proposed microsomal components eventually will be required to validate their independent existence.

Acknowledgment—We wish to thank Mrs. Miriam Dixon for valuable technical assistance.

REFERENCES

Enzymatic Aromatization of Steroids: I. EFFECTS OF OXYGEN AND CARBON MONOXIDE ON THE INTERMEDIATE STEPS OF ESTROGEN BIOSYNTHESIS
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