Biogenesis of Estrogens by the Human Ovary*

III. CONVERSION OF CHOLESTEROL-4-C\textsuperscript{14} TO ESTRONE

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Although cholesterol has been accepted as a precursor of the neutral steroid hormones, its role in estrogen formation has been subject to much doubt. It was postulated, on the basis of experiments in vivo in the pregnant mare, that cholesterol was not an estrogen precursor (1). The test animal had readily converted radioactive acetate to labeled estrogens, but had failed to convert administered radioactive cholesterol. Furthermore, in the acetate experiment, the serum cholesterol was not significantly labeled. At that time, it was felt that the conversion of neutral steroids to estrogens was not an important pathway, thereby excluding a major precursor role for cholesterol as well (1). A discussion of the validity of these arguments has been published (2).

The conversion of neutral steroids to estrogens has now been shown to be, in fact, a quantitatively significant reaction (3, 4), and the conversion in vivo of cholesterol to estrone in a pregnant patient has been demonstrated, although in extremely low yield (5).

This is the third of a series of papers on the biogenesis of estrogens by the human ovary (cf. (4, 6)). It had been demonstrated in an earlier study, that acetate-1-C\textsuperscript{14} was converted to cholesterol as well as to estrogens in amounts consistent with a possible precursor role for cholesterol (6), and that progesterone, itself a cholesterol metabolite (7), could be converted to estrogens in 10% yield (4). In this report, the conversion in vitro of cholesterol-4-C\textsuperscript{14} to estrone by the human ovary is described.

EXPERIMENTAL PROCEDURE

Tissue Preparation and Incubation—A normally menstruating patient scheduled for laparotomy was treated with 175 mg of ovine FSH\textsuperscript{A} over a 9-day period before surgery. The FSH was generously supplied by the Endocrinology Study Section, National Institutes of Health. Both ovaries (47.5 g) were obtained at surgery, dissected and the follicular linings isolated. The follicular cyst linings (1,975 g) were minced and suspended in chilled Krebs' phosphosaline buffer. The tissue was incubated for 4 hours at 37\(^\circ\)C with 1 \(\mu\)c of cholesterol-4-C\textsuperscript{14} (37.7 \(\mu\)c per mg) (New England Nuclear Corporation) in Krebs' phosphosaline buffer at pH 7.4.

Measurements of Radioactivity and Estrogen Assay—Radioactivity was measured in duplicate as previously described (6) and estrogen analyses were carried out in duplicate by the sulfitographic procedure (8). Duplicate for all assays agreed within 5%.

Gradient elution partition chromatography was carried out on Celite by the method described by Engel et al. (9). The method used for the formation of estrone methyl ether and its subsequent purification has been reported by Brown (10). Paper chromatography was performed at room temperature with the procedure of Bush (11) with solvent systems noted in the text. The estrone methyl ether was crystallized from ethanol.

RESULTS

Extraction—The incubation mixture was processed as previously described (6). Three volumes of hot alcohol and 25 volumes of hot acetone were added in small amounts to the incubation flask and the mixture was filtered. The remaining tissue was ground with sand, re-extracted with acetone, and filtered. The combined alcohol-acetone was evaporated to dryness under reduced pressure, and the residue was taken up in 5 ml of water and extracted 3 times with 6 volumes of chloroform. The chloroform was evaporated and the residue partitioned between equal volumes of pentane and 90% methanol. The pentane fraction contained 81% of the starting radioactivity (1.2 \(\times\) 10\(^6\) c.p.m.) and the 90% methanol fraction contained 4% of the starting radioactivity (5.8, 385 c.p.m.). The 90% methanol fraction was next separated into a neutral fraction (45,070 c.p.m.), an "estrone-estradiol" fraction (6,177 c.p.m.), and an "estriol" fraction (4,384 c.p.m.) by the method of Brown (10) as described in an earlier report of this series (6). Only the "estrone-estradiol" fraction was processed further.

Purification of "Estrone-Estradiol" Fraction—The fraction designated the "estrone-estradiol" fraction (6,177 c.p.m.) was subjected to an 8-transfer countercurrent distribution in separatory funnels between toluene and \(\times\) NaOII according to the procedure of Baggett et al. (12). Half of the applied radioactivity was concentrated in the higher numbered tubes which contained the phenolic fraction (1,987 c.p.m.). These tubes were pooled and applied to a Celite column with estrone, estradiol, and estriol carriers (100 \(\mu\)g of each). The stationary phase was 90% methanol and the moving phase was trimethylpentane with a gradient of trimethylpentane-ethylene dichloride (1:1) after the first 36 tubes had been collected. Alternate tubes from the column were counted and in the areas of concentration of counts each tube was counted for radioactivity and assayed for estrogens. Total recovery of radioactivity from the column was 87.5% and recovery of carrier estrone and estradiol was 93.7% and 98.5%, respectively.

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1 The abbreviation used is: FSH, follicle-stimulating hormone.
The distribution of carrier estrone and radioactivity are illustrated in Fig. 1. The peak tube for estrogen fluorescence and counts per minute coincided and the curves for radioactivity and carrier estrone were superimposable. Of the applied radioactivity 47.7% (947 c.p.m.) was recovered with the carrier estrone.

The carrier estradiol was recovered in a sharp peak after the gradient was applied. A curve of radioactivity (517 c.p.m.) was obtained which did not coincide with the carrier estradiol curve. This metabolite has not been identified. After the estradiol was recovered from the column, a gradient of ethylene-dichloride was applied and the carrier estradiol was recovered. There was no radioactivity in this fraction, as would be expected, inasmuch as any more polar steroids should have been removed in the original extraction of the benzene-petroleum ether with water ("estriol" fraction).

**Purification of Estrone**—The estrone pool recovered from the Celite column (383 c.p.m.; 74.5 μg of estrone) was next saponified (13) without change in specific activity. The material recovered from the saponification was methylated, purified by treatment with hydrogen peroxide and partition between petroleum ether and sodium hydroxide, and subjected to alumina chromatography (10) with a resultant slight drop in specific activity. Recrystallized estrone methyl ether (5 mg) (m.p., 171-172°) was next added as carrier and the mixture was recrystallized to constant specific activity. Both crystals and mother liquors were assayed for estrogen fluorescence and counts per minute. A summary of the purification of the estrone is presented in Table I. As can be noted from the specific activity of the first mother liquor, a slight purification was achieved with the first crystallization. Thereafter, the specific activities of the crystals and mother liquors were essentially identical.

**Control Experiments**—An amount of cholesterol-4-C14 equivalent to the starting material was processed in an identical manner without prior incubation and divided into a neutral fraction, an "estrone-estradiol" fraction, and an "estriol" fraction as described above. The "estrone-estradiol" fraction was subjected to an 8-transfer countercurrent distribution between toluene and 0.5% NaOH and the pooled phenolic portion was counted for radioactivity and chromatographed on a toluene-75% methanol system. It was established that the starting cholesterol was not contaminated with either estrone or estradiol. The neutral fraction was subjected to a Girard separation (14) and paper chromatography on a ligroin-96% methanol system, and it was established that the estrogen was not contaminated with other neutral compounds which could serve as estrogen precursors.

**Assay of Endogenous Estrogens in Follicular Fluid**—Before dissection of the ovaries, the follicular cysts were aspirated of 28 cc of clear, straw-colored fluid. This was extracted and assayed for estrogens by the method of Smith (15). This included addition of radioactive estrone and estradiol for reverse isotope dilution, purification on a Celite column, methylation, purification of the methyl ethers, and alumina chromatography (15). Estrone (23.1 μg/100 ml of follicular fluid) and estradiol (62.8 μg/100 ml of follicular fluid) were isolated.

Concentrations of the same order of magnitude have been recovered from follicular cyst fluid and corpora lutea of patients not treated with FSH (15-17).

**DISCUSSION**

Although it had been thought that the biogenesis of estrogens involved a completely distinct pathway (1), there is now increasing evidence that the estrogens share common precursors with the adrenal and testicular steroid hormones. The present study lends support to the concept that cholesterol can be a precursor of the estrogens and confirms in vivo the role of progesterone as an estrogen precursor has been demonstrated in an earlier report of this series (4).

As in earlier studies from this laboratory, FSH was used to enhance the enzymatic activity of human ovaries before their surgical removal. In this manner, the rather elusive conversion of cholesterol to estrogens could be demonstrated. The procedures followed for establishing identity of the radioactive metabolites were similar to our previous studies (4, 6) and included the fine resolution of Celite chromatography and the relative specificity of methyl ether formation and purification. Finally, crystallization to constant specific activity was achieved.

![FIG. 1. The estrone curve from the Celite column. The stationary phase was 90% methanol; the moving phase was trimethylpentane. ●●● counts per minute; ○○○ fluorescence. Each tube represents 1 column volume.](http://www.jbc.org/)

**TABLE I**

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Radioactivity</th>
<th>Amount of estrone</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pool from Celite column</td>
<td>933</td>
<td>74.5</td>
<td>12.5</td>
</tr>
<tr>
<td>Saponification</td>
<td>952</td>
<td>76.7</td>
<td>12.4</td>
</tr>
<tr>
<td>Methylation, treatment with H2O2, and petroleum ether- NaOH partition</td>
<td>896</td>
<td>77.5</td>
<td>11.6</td>
</tr>
<tr>
<td>Alumina chromatography</td>
<td>726</td>
<td>72</td>
<td>10.1</td>
</tr>
<tr>
<td>Estrone methyl ether carrier added (5 mg)</td>
<td>630</td>
<td>4.2</td>
<td>150</td>
</tr>
<tr>
<td>First crystallization</td>
<td>492</td>
<td>3.1</td>
<td>159</td>
</tr>
<tr>
<td>Second crystallization</td>
<td>108</td>
<td>0.65</td>
<td>166</td>
</tr>
<tr>
<td>Mother liquor</td>
<td>440</td>
<td>2.9</td>
<td>152</td>
</tr>
<tr>
<td>Third crystallization</td>
<td>335</td>
<td>2.5</td>
<td>153</td>
</tr>
<tr>
<td>Mother liquor</td>
<td>88</td>
<td>0.988</td>
<td>149</td>
</tr>
</tbody>
</table>
In contrast to prior work in which acetate and progesterone were used as substrates (4, 6), no estradiol was recovered in this study which utilized cholesterol as an estrogen precursor. The significance of this observation is not readily apparent because the factors which control the estrone-estradiol ratio in a given tissue are largely unknown. The complex interplay between testosterone and androstenedione and estrone and estradiol which is a result of the 17β-ol dehydrogenases has been mentioned in a report on estrogen formation by the human placenta (3).

It is interesting to note that the present conversion of cholesterol to estrone was approximately 0.1% by isotope dilution whereas in earlier studies the conversion of acetate to estrogens was 0.02% and the conversion of progesterone to estrogens was 10% (4, 6). This provides an order of ascending yield as one starts with a substrate biosynthetically closer to the end product. It is hoped that further studies of these reactions will provide greater insight into the rate-controlling steps in estrogen biosynthesis.

SUMMARY

The conversion in vitro of cholesterol-4-C14 to estrone by human ovarian tissue has been described. Criteria for the identity of the radioactive metabolite and complete data on recovery and changes in specific activity have been provided.

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