The Conversion of Testosterone-3-C\textsuperscript{14} to C\textsuperscript{14}-Estradiol-17\beta by Human Ovarian Tissue*

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There is a considerable body of biological data linking testosterone and other 19-carbon steroids with the estrogens. Both ovaries and testes may, under certain circumstances, produce androgens and estrogens (1-4). The administration of testosterone and other androgens to human subjects leads to an increased urinary excretion of estrogenic material (5-10), which has been identified as estrone, estradiol, and estriol (11). These observations suggest a possible conversion of 19-carbon steroids to phenolic estrogens.

Studies on the biosynthesis of steroid hormones have indicated that cholesterol can serve as a precursor of progesterone (12) and the adrenal cortical steroids (13). There is, however, some evidence that cholesterol is not a necessary intermediate in these biological syntheses (14). The conversion of cholesterol to estrogenic steroids has not been demonstrated. Heard et al. isolated C\textsuperscript{14}-estrone from the urine of a pregnant mare to which CH\textsubscript{3}C\textsuperscript{14}-OONa was administered intravenously (15), but found no C\textsuperscript{14} in the urinary estrone after cholesterol-4-C\textsuperscript{14} was given by the same route (16).

Recently Heard et al. have reported the excretion of C\textsuperscript{14}-labeled estrone by a pregnant mare to which C\textsuperscript{14}-labeled testosterone was administered (17).

This paper reports the results of an experiment in vitro carried out to study the possible biochemical conversion of testosterone to estrogenic steroids by human ovarian tissue.

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EXPERIMENTAL

Determination of Radioactivity—All samples for radioactivity analysis were plated directly in stainless steel counting dishes (1 inch in diameter, \(\frac{1}{8}\) inch in depth). The counts were then determined in a Tracerlab SC-16 windowless flow counter operated in the Geiger region. All counts are at infinite thinness with no more than 1 mg. being plated in any instance.

Determination of Estrogens—The counter-current distributions were analyzed for estrogen by the method of Engel et al. (18), based on fluorescence in \(\text{H}_2\text{SO}_4\). A Farrand photofluorometer was employed for the measurement of fluorescence.

Tissue Incubation—Both ovaries were removed surgically from a 34 year-old woman with cervical carcinoma. The operation took place near the middle of a menstrual cycle. Grossly the ovaries appeared normal, and one contained a well developed follicle.

The ovaries were sliced by hand to a thickness of about 0.5 mm. and added to the incubation medium in a 125 ml. flask. The medium consisted of 20 ml. of human plasma, to which had been added 90 mg. of glucose, 0.18 mmole of citric acid, 0.02 mmole of sodium fumarate, 500 units of chorionic gonadotropin (A. P. L.; Ayerst, McKenna and Harrison), 500 units of equine gonadotropin (Equinex; Ayerst, McKenna and Harrison), and 0.23 mg. of testosterone-3-C\(^14\) (total of 550,000 c.p.m.), previously dissolved in 0.1 ml. of propylene glycol. The flask was filled with \(\text{O}_2\), closed, and incubated at 37.5\(^\circ\) with shaking for 3 hours. At the end of the incubation period, the mixture was frozen and kept until processed.

Extraction and Preliminary Fractionation—The tissue and medium were thawed, and 2 mg. each of estrone, estradiol,\(^1\) and estriol were added as carriers. 2 volumes of hot acetone were added, and the mixture was shell-frozen and dried from this state. The dry tissue was extracted for 4 hours in a Soxhlet extractor with chloroform.

The chloroform was removed \textit{in vacuo}, and the extract was dissolved in 50 ml. each of pentane and 90 per cent methanol. These solvents were equilibrated, and the 90 per cent methanol layer was removed. The pentane was extracted three additional times with 50 ml. of 90 per cent methanol. The pentane and methanol fractions were evaporated \textit{in vacuo}. The residue from the methanol fraction (335,000 c.p.m.) was dissolved in 50 ml. of toluene, and this solution was extracted four times with 12 ml. of \(\text{N}\) \(\text{NaOH}\) and twice with 12 ml. of \(\text{H}_2\text{O}\). The combined alkali and water extracts were adjusted to pH 8.5, and the mixture was extracted three times with 45 ml. of diethyl ether (18). The residue, after evaporation of the ether, contained 5600 c.p.m. The neutral material, after removal of the toluene, contained 262,000 c.p.m.

\(^1\) Estradiol as used throughout this paper refers to estradiol-17\(\beta\).
Counter-Current Distribution between Toluene and 1 N NaOH—The phenolic fraction obtained from the preliminary fractionation was subjected to an eight transfer counter-current distribution between toluene and 1 N NaOH, 20 ml. in each layer (19). Separatory funnels were used and the lower layer was transferred.

When eight transfers had been completed, the pH of all the lower layers was adjusted to 8.5 to 9.0. The two layers in each funnel were reequilibrated, thereby extracting phenolic material into the toluene. Each lower layer was then reextracted twice with 10 ml. of diethyl ether. The combined toluene and ether extracts from each funnel were washed once with 3 ml. of H₂O and then evaporated to dryness. Aliquots were removed from each fraction for counting and for analysis for estrogens.

Fig. 1 shows the distribution of radioactivity in this counter-current distribution. There is a striking peak in activity in tube 8, indicating the presence of C¹⁴-labeled phenolic material. Analysis for estrogens showed that they, too, were concentrated in tubes 5 to 8, with the peak in tube 8. When a sample of the testosterone-3-C¹⁴ used in this experiment was subjected to a similar eight transfer distribution, no peak in radioactivity was found in the higher numbered tubes.

99 Transfer Counter-Current Distribution—As the next step in the fractionation of the phenolic material, the contents of tubes 5 to 8 from the eight transfer distribution were pooled and subjected to a 99 transfer counter-current distribution in 30 per cent ethyl acetate-70 per cent cyclohexane with 50 per cent ethanol-50 per cent H₂O as the lower phase. The distribution was carried out in a 100 tube Craig machine (H. O. Post Scientific Instrument Company). All fractions from the distribution were evaporated in vacuo from the frozen state.

Aliquots were taken from selected tubes throughout the distribution for
determination of radioactivity and for analysis for estrogen. The results of these analyses are seen in Fig. 2. The scales of the ordinate for radioactivity and the ordinate for weight of estrogen were selected so that the peak in radioactivity in the estradiol region was the same height as the peak in weight.

This counter-current distribution shows that there is no significant C\textsuperscript{14} in the estrone region (E\textsubscript{1}). There is, however, a peak in radioactivity coincident with the peak in weight of estradiol (E\textsubscript{2}). The experimental values for weight and for radioactivity fit a single theoretical distribution curve equally well, thus indicating that the estradiol contained C\textsuperscript{14}. There is also a peak in radioactivity in the same tube as the peak in weight of estriol (E\textsubscript{3}). The difference in the height of the weight and radioactivity peaks is a result of the arbitrary adjustment of the two ordinates. It appeared likely, therefore, that the estriol was also labeled.

**Redistribution of Estriol**—The contents of tubes 2 to 14 (estriol region) of the preceding counter-current distribution (Fig. 2) were pooled. 4 mg. of additional carrier estriol were added. The estriol was then subjected to a forty-nine transfer counter-current distribution in 70 per cent aqueous methanol with 80 per cent CHCl\textsubscript{3}-20 per cent CCl\textsubscript{4} as the lower phase and was analyzed as described above. The radioactivity was separated from

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**Fig. 2.** 99 transfer counter-current distribution separating the three estrogens. Upper layer is 30 per cent ethyl acetate-70 per cent cyclohexane; lower layer, 50 per cent aqueous ethanol. Upper layers were transferred. In Figs. 2, 3, and 4, the following symbols are used: $K$, the partition coefficient (ratio of concentration in upper layer to concentration in lower layer); $O$, experimental points for weight of estrogen referred to the ordinate on the left; $\bullet$, counts per minute referred to the ordinate on the right; solid lines, the best calculated theoretical curves through the experimental points for weight. $E\textsubscript{1}$ = estrone, $E\textsubscript{2}$ = estradiol-17\beta, $E\textsubscript{3}$ = estriol. $K$ for $E\textsubscript{1}$ = 2.00, for $E\textsubscript{2}$ 0.94, for $E\textsubscript{3}$ 0.076.
the carrier estriol by this distribution, the peak for weight of estriol being in tube 25, and that for radioactivity in tube 0.

Redistribution of Estradiol—The contents of tubes 34 to 56 of the 99 transfer distribution (Fig. 2) were pooled. The pool contained 0.34 mg. of estradiol and 1150 c.p.m. (3380 c.p.m. per mg.). An additional 10.0 mg. of carrier estradiol were added, which brought the specific activity to 112 c.p.m. per mg. The estradiol was then subjected to a forty-nine transfer counter-current distribution in 70 per cent aqueous methanol with 40 per cent CHCl₃-60 per cent CCl₄, as the lower phase. The results of the analyses of this distribution are shown graphically in Fig. 3. Again, the distribution of radioactivity and weight of estradiol fit the same theoretical curve. Calculation of the area under the theoretical curve showed the presence of 7.4 mg. and 695 c.p.m., thus giving a specific activity of 94 c.p.m. per mg. Calculation of specific activities for the individual tubes under the peak gave a value for the specific activity of 87 ± 8 (standard error of the mean, fifteen determinations).

Precipitation with Digitonin (20)—The contents of tubes 14 to 30 from the forty-nine transfer counter-current distribution (Fig. 3) of the estradiol were pooled. The estradiol was dissolved in 0.9 ml. of hot methanol with 30 mg. of digitonin. Hot water was added until the solution was faintly cloudy (0.3 ml.), and, after standing for an hour at room temperature, an additional 0.3 ml. of H₂O was added. While the solution was left to stand at 5° for 3 hours, a flocculent precipitate separated. The precipitate was centrifuged and washed twice with cold 60 per cent methanol, redissolved in 1.5 ml. of hot 60 per cent methanol, and again allowed to precipitate in the cold. This precipitate was centrifuged and washed with cold 60 per cent methanol. The mother liquors from the first and second precipita-
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Titions were kept separate. The residues from the two mother liquors and the precipitate were each dissolved in 1 ml. of anhydrous pyridine and heated on the steam bath for 1 hour. 10 ml. of absolute ether were added to each fraction, and the digitonin was removed by centrifugation. The ether solutions were washed twice with 1 ml. of H₂O and then evaporated to dryness in vacuo. Each fraction was counted and analyzed for estradiol by the fluorescence method. The first mother liquor contained 1.08 mg. of estradiol and 128 c.p.m. (119 c.p.m. per mg.). The second mother liquor contained 0.74 mg. of estradiol and 67 c.p.m. (91 c.p.m. per mg.); the final precipitate contained 2.98 mg. of estradiol and 276 c.p.m. (93 c.p.m. per mg.). In this purification procedure, therefore, there was no tendency for the radioactivity to separate from the estradiol.

Enzymatic Oxidation of Estradiol—Since there was no indication of a separation of radioactivity from the estradiol in the preceding step, the estradiol from the mother liquors and precipitate was pooled. The crystalline residue was slightly brown. To remove this brown color another phenolic separation was carried out. The neutral fraction contained no radioactivity; the estradiol was colorless and crystalline.

This estradiol was then incubated for 3 hours at 25° in the presence of oxidized diphosphopyridine nucleotide with a partially purified preparation of placental estradiol-17β dehydrogenase. This enzyme has a high degree of specificity for the reversible oxidation of estradiol-17β to estrone.

After incubation, the mixture was extracted four times with 0.5 volume of methylene chloride. The residue from this extract was subjected to a phenolic separation. The phenolic fraction was then subjected to a twenty-four transfer counter-current distribution in 50 per cent methanol-carbon tetrachloride. A stainless steel machine with twenty-five tubes was utilized in this distribution (H. O. Post Scientific Instrument Company). The results of this distribution are shown in Fig. 4. Practically all of the estradiol (K = 2.45) has been oxidized to estrone (K = 0.33). The radioactivity was associated with the estrone. A single theoretical curve fits the data for radioactivity and weight of estrone. The distribution showed the presence of 2.28 mg. of estrone with 228 c.p.m. (100 c.p.m. per mg.). Calculation of specific activities of individual tubes under the peak gave a value of 101 ± 5 (standard error of the mean, nine determinations) c.p.m. per mg. The estrone from this distribution was pooled and recrystallized from aqueous ethanol. The recrystallized estrone weighed 1.14 mg. and contained 106 c.p.m. (93 c.p.m. per mg.).

Summary of Specific Activities—Table I shows the weight of estradiol

* We wish to express our sincere appreciation to Miss Lorna Langer of the Huntington Laboratories for furnishing this enzyme preparation and for information on its properties and use.
determined by fluorescence in H₂SO₄, the counts per minute in the estradiol, and the resulting specific activity at each step in the purification of the estradiol. It is apparent that the specific activity remained essentially constant throughout all purification steps subsequent to the 99 transfer counter-current distribution.

![Graph showing the transfer counter-current distribution of oxidized estradiol. The upper layer is 50 per cent methanol, lower layer CCl₄. The upper layers were transferred. See Fig. 2 for an explanation of the symbols.](image)

**Table I**

<table>
<thead>
<tr>
<th>Step in purification</th>
<th>Weight, mg.</th>
<th>C.p.m.</th>
<th>C.p.m. per mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>99 transfer counter-current distribution</td>
<td>0.34</td>
<td>1150</td>
<td>3380</td>
</tr>
<tr>
<td>99 “” “” + 10 mg. added carrier</td>
<td>10.3</td>
<td>1150</td>
<td>112</td>
</tr>
<tr>
<td>49 transfer counter-current distribution</td>
<td>7.4</td>
<td>695</td>
<td>94</td>
</tr>
<tr>
<td>Regenerated from digitonide</td>
<td>2.98</td>
<td>276</td>
<td>93</td>
</tr>
<tr>
<td>Oxidized to estrone</td>
<td>2.28</td>
<td>228</td>
<td>100</td>
</tr>
<tr>
<td>Recrystallized estrone</td>
<td>1.14</td>
<td>106</td>
<td>93</td>
</tr>
</tbody>
</table>

These results, therefore, strongly indicate the radiochemical purity of the estradiol. A conversion of 1.2 per cent of the testosterone had occurred as calculated by isotopic dilution.

Neutral Compounds—The neutral fraction from this incubation of testosterone-3-C³⁴ with ovarian tissue was fractionated by paper chromatography (21). Approximately 70 per cent of the activity was present as unchanged testosterone. Most of the remaining radioactivity was present in a compound whose mobility on paper chromatograms was consistent with Δ⁴-androstene-3,17-dione. A small fraction of the radioactivity could not be moved from the starting lines.
DISCUSSION

The methods employed for the isolation and radiochemical purification of the estradiol have been selected as those most likely to remove possible labeled contaminants. The eight transfer distribution between alkali and toluene represents a systematic method for carrying out extraction procedures so as to determine whether a separation has taken place or whether the same material is present in both fractions. In this particular case, the original phenolic fraction contained only a small fraction of the total radioactivity. There was some doubt as to whether any of this radioactivity was really present in phenolic compounds, since some neutral material would certainly be partitioned into the aqueous phase and might account for all of the radioactivity in that fraction. The eight transfer counter-current distribution showed a definite separation into two fractions, thus indicating that a large percentage of the activity in the phenolic fraction differed from the bulk of neutral material. In an earlier experiment similar to the one described here, labeled testosterone was incubated with slices of stallion testis. The eight transfer counter-current distribution was not used, and the estrone separated from the phenolic fraction contained radioactivity. After more extensive purification, the radioactivity was separated and shown to reside in testosterone present as a contaminant. An eight transfer distribution of the type described here would have eliminated this more tedious purification.

Counter-current distribution seems to be a particularly valuable method for studies of radiochemical purity; it offers a precise method for the determination of a partition coefficient with the opportunity for individual determinations which act as repeated analytical checks, one for each tube analyzed. If the radioactivity and weight of the carrier both have peaks in the same tube of a distribution, both must have the same partition coefficient within very narrow limits. Interaction between the carrier and a labeled contaminant is not likely since very dilute solutions are used in distributions. In order to detect a lack of radiochemical purity, a marked separation of carrier from a labeled contaminant is not required. If peaks in weight of carrier and in radioactivity are separated only by a single tube, this inhomogeneity is often apparent since different theoretical curves will be required to fit the two sets of data. The specific activity will vary from tube to tube in a systematic manner. Of course, the greater the number of distributions in different solvent systems carried out with failure to demonstrate a lack of homogeneity, the greater is the degree of certainty that the radioactivity resides in the carrier.

8 Unpublished data.
The precipitation of estradiol as the digitonide without a change in specific activity is good evidence that the label is present in the estradiol. It would be expected that a radioactive impurity in low concentration would not be precipitated in appreciable amounts, even though in higher concentrations it might form the insoluble complex.

The oxidation of the labeled estradiol to estrone represents a chemical conversion employed to supplement the physical criteria for radiochemical purity. This conversion was carried out enzymatically so as to offer additional specificity to the reaction.

It would seem quite unlikely that a labeled impurity could accompany the carrier estradiol through such rigorous purification steps. The C\(^{14}\) almost certainly resides in the estradiol molecule.

Since the labeled carbon atom has not been located in the estradiol, the possibility of breakdown of the testosterone to small fragments, followed by synthesis of estradiol from the labeled fragments, must be considered. The recovery of most of the radioactivity in unchanged testosterone eliminates extensive breakdown. If breakdown did occur to some extent, considerable dilution of the fragments would be expected from other metabolic processes. It seems unlikely that as large a fraction of the total radioactivity as 1.2 per cent could find its way into estradiol by this pathway.

This conversion is of particular interest, since it involves profound changes in the molecule, loss of the angular methyl group, and aromatization. The mechanism for such a conversion is worthy of further study. The hydroxylation of carbon 19 may serve as the initial reaction which leads to the removal of this carbon atom. Meyer has recently demonstrated the conversion of Δ\(^{4}\)-androsten-19-ol-3,17-dione to estrone by endocrine tissue (22). The formation of Δ\(^{4}\)-androsten-19-ol-3,17-dione from androstenedione has been demonstrated in adrenal tissue (23). The introduction of a second double bond may also be an intermediate reaction. The loss of formaldehyde and the aromatization of a steroid containing a 19-hydroxy-3-keto-1,4-diene grouping has been demonstrated under mildly alkaline conditions (24).

The rôle of the conversion of testosterone to estradiol in normal biosynthesis of the estrogens is not certain. This conversion could well account for the increased excretion of estrogens following the administration of testosterone; however, this increased excretion has been observed in castrated humans (9, 11) and in an adrenalectomized, castrated human (25). Hence, the rôle of endocrine tissue in the conversion may be questioned. However, in another incubation study carried out in a manner similar to the one presented here, ovaries removed from a woman early in a menstrual cycle failed to carry out the conversion. In other experi-
ments, the conversion of testosterone to estradiol has been demonstrated to be carried out by stallion testes, a known source of estrogenic steroids, and by a feminizing adrenal tumor. In addition to labeled estradiol, labeled estrone was also formed by the tumor. These few correlations of the conversion with known sources of estrogen biosynthesis are suggestive of the importance of the conversion in the normal biosynthetic pathway.

SUMMARY

C\textsuperscript{14}-labeled estradiol-17\beta has been isolated following the incubation of testosterone-3-C\textsuperscript{14} with human ovarian slices. \(\Delta^4\)-Androstene-3,17-dione was the only other compound identified as a conversion product.

The authors wish to express their sincere appreciation to Dr. Howard Ulfelder and Dr. Joe V. Meigs for furnishing the human tissue used in this study. They also wish to thank Dr. Marcel Gut for the preparation of the labeled testosterone, prepared from BaC\textsuperscript{14}O\textsubscript{3} obtained on allocation from the United States Atomic Energy Commission. For a generous gift of Equinex and A. P. L., the authors are grateful to Ayerst, McKenna and Harrison, Ltd.

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