Deuterium-labeled Steroids for Study in Humans

I. ESTROGEN PRODUCTION RATES IN NORMAL PREGNANCY*

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SUMMARY
Deuterium-labeled 1,3,5(10)-estratriene-3,17β-diol was used for the estimation of estrogen production rates during normal pregnancy. The results for three patients are concordant with the available reports in the literature. Isotope dilution data were obtained by gas chromatography-mass spectrometry. The steroids were monitored by conventional scanning of the molecular weight range at 20 eV or 70 eV and at selected m/e values at 20 eV by the use of an accelerating voltage alternator.

Recent advances in gas chromatographic-mass spectrometric techniques have made it reasonable to reinvestigate the use of stable isotopes, for example deuterium (1-3), to study the fate of steroid hormones in human subjects. The use of deuterated compounds in contrast to compounds labeled with radioactive isotopes allows repeated clinical studies on a pregnant or non-pregnant patient which otherwise, for ethical reasons, would be impossible to perform.

The purpose of this study was to determine whether the isotope dilution technique, with a deuterium-labeled steroid, was applicable for the estimation of estrogen production rates during normal pregnancy.

EXPERIMENTAL PROCEDURE
General—All reagents were of analytical grade. Solvents were freshly distilled to gas chromatographic purity. Commercially available previously coated thin layer chromatography plates (20 x 20 cm) of two different layer thicknesses (250 and 500 μ) from Analtech, Inc., Wilmington, New Jersey were first washed with methanol for 24 hours and then with benzene for 24 hours and were activated at 115° for 30 min before use. Deuterium-labeled estradiol-17β was prepared by catalytic hydrogenation of 4-bromoestradiol-17β (4) with deuterium gas in alkaline methanol (5). Confirmation of the purity of the compound was established by thin layer and gas chromatography and by mass spectrometry. Mass spectral analysis of the labeled product indicated a mole per cent distribution of 10.8% for the unlabeled species d0, 83.8% for the singly labeled species d1, and 5.4% for the doubly labeled species d2.

Three healthy pregnant women were studied (Table I). Estradiol-17β-4-2H was administered into the antecubital vein over a 5-min period in 2 to 3 ml of a sterile propylene glycol-water (3:1) mixture. Total 24-hour urine collections were instituted for 2 days prior to administration of the steroid and continued for 7 days. The following urine samples were pooled: control urines, deuterium-containing urines (4-day pool), Day 5 urine, and late urine (Days 6 and 7). Urine specimens were refrigerated during collection until brought to the laboratory where they were immediately processed or kept in a deep freeze at -15°C.

Extraction of Urinary Conjugates (6) Aliquots (25%) in duplicate of the pools (average 24-hour total volume) and of individual 24-hour specimens were adjusted to obtain a final concentration of 3.0 m sodium chloride and 0.25 m dipyrinidium sulfate while diluting with an equal volume of water. Conjugates were extracted with ethyl acetate, once with an equal volume and twice with half-volumes. After drying with anhydrous sodium sulfate, the combined extract was evaporated in a vacuum below 45°C.

Enzymic Hydrolysis of Urinary Conjugates—The residue of urinary conjugates was dissolved in 50 ml of 0.1 m acetate buffer, pH 5.2. Glusulase (Endo Labs, Garden City, New York) was added (52,000 modified Fishman units of β-glucuronidase and 14,000 units of sulfatase), and the solution was incubated at 37° for 3 days.

Extraction and Partition of Free Estrogens—The hydrolysate was extracted with ether (3 x 50 ml), and the combined extracts were washed with 15 ml of 8% sodium bicarbonate and 8 ml of water and then dried over anhydrous sodium sulfate. After evaporation in a vacuum, 1 ml of purified absolute alcohol was added to dissolve the residue and was followed by the addition of 30 ml of benzene. The solution was transferred to a separatory funnel containing 30 ml of hexane, and the mixture was extracted with water (3 x 10 ml) (estriol + 16-epi-estriol fraction) followed by 1 ml sodium hydroxide (3 x 10 ml) (estrone + estratriol fraction). Sodium bicarbonate (2 g) was added to the estriol + 16-epi-estriol fraction which was extracted with ether (3 x 15 ml). The ether extract was washed with water (2 x 3 ml), dried over anhydrous sodium sulfate, and evaporated to dryness under nitrogen. The sodium hydroxide extract was neutralized to pH 7 to 9 with 6 N hydrochloric acid. Subsequent to the
was carried out with a model 7401 instrument (Packard Instrument Company) on glass columns (6 feet x 4 mm inner diameter) packed with 3% OV-101 on 100 to 120 mesh Supelcoport. Carrier gas (nitrogen) flow rate was 40 ml per min. The temperatures were for E1 (retention time 9.8 min) or E2 (retention time 12.0 min) were as follows: flash heater, 270°, detector, 270°, and column, 245°. For E3 (retention time 10.8 min) and 16-Epi-E3 (retention time 11.6 min), the temperature was 290°.

Thin Layer Chromatography on Silica Gel—The following solvent systems were used for thin layer chromatography: System 1, benzene-acetone (3:2); System 2, benzene-ethyl acetate (1:1); System 3, hexane-methylene chloride-absolute ethanol (10:9:1); System 4, chloroform-acetone (3:2). Thin layer plates of 500 μ thickness were developed with Systems 1, 2, and 4 in incompletely saturated chambers at room temperature (22°), whereas those of 250 μ thickness were developed with System 3 in saturated (inner wall lined with filter paper) chambers at 4°.

Estrogen residues were dissolved in 100 μl of acetone-benzene (7:3) mixture and applied to the thin layer chromatography plates as 3- to 5-cm streaks. The process was repeated twice with 50-μl portions of solvent mixture. Two samples were applied to each plate whereas standard solutions of estrogens were applied between the sample streaks and near the outer ends. Standards were visualized after development by spraying with ammonia-air stream. Silica gel from the areas corresponding to the standards was removed by aspiration into silanized glass wool filters packed in disposable Pasteur pipettes previously washed with the eluting solvent. Elution was performed with a mixture of methylene chloride-methanol (9:1). The eluates were evaporated to dryness under a nitrogen stream at 55°.

Estrone and estradiol-17β were separated in System 1 and purified further in System 3. Estriol and 16-epi-estriol were separated in System 2 and purified further in System 4.

Derivative Formation and Gas Chromatography—Isolated estrogens were dissolved in a mixture of 0.10 ml of anhydrous pyridine, 0.10 ml of hexamethyldisilazane, and 0.05 ml of trimethylchlorosilane. The tubes were capped and heated at 65° for 1 hour. The reagents were evaporated at 50–55° under a stream of dry nitrogen. The derivatives were dissolved in anhydrous hexane before injection into the gas chromatograph. Gas chromatography prior to combined gas chromatography-mass spectrometry was carried out with a model 7401 instrument (Packard Instrument Company) on glass columns (6 feet x 4 mm inner diameter) packed with 3% OV-101 on 100 to 120 mesh Supelcoport. Carrier gas (nitrogen) flow rate was 40 ml per min. The temperatures for E1 (retention time 9.8 min) or E2 (retention time 12.0 min) were as follows: flash heater, 270°, detector, 270°, and column, 245°. For E3 (retention time 10.8 min) and 16-Epi-E3 (retention time 11.6 min), the temperature was 290°.

Mass Spectrometry—Gas chromatography-mass spectrometry was performed with a LKB-9000 instrument (LKB Produkter AB, Stockholm, Sweden) operated at an ionizing voltage of 70 e.v., an ionizing current of 60 μA, and an accelerating voltage of 3.5 kv. The ion source temperature and the Becker-Ryhage separator (8) were maintained at 250°. The column used was 3.5 kv. The ion source temperature and the Becker-Ryhage separator (8) were maintained at 250°. The coiled glass column (6 feet x 2 mm inner diameter) was packed with 3% OV-101 on 100 to 120 mesh Supelcoport and maintained at 245°. The most abundant steroidal ion in the mass spectrum for each estrogen was the molecular ion. The molecular ion for estrone trimethylsilyl ether was the base peak, whereas for the trimethylsilyl ethers of estradiol-17β, estriol, and 16-epi-estriol the base peak was at m/e 73. The molecular ions were found to be the most useful for the calculation of the relative abundance of deuterated and nondeuterated species. Mass spectra were obtained at the apex of the total ion current curve.

Because of the possibility of fractionation of deuterated and nondeuterated steroids during gas chromatography (9), an investigation was undertaken involving gas chromatography-mass spectrometry analysis of labeled reference standard and biological samples both by conventional scanning at 70 e.v. and 20 e.v. and by the accelerating voltage alternator (20 e.v.) at selected m/e values, e.g. [M]+, [M + 1]+, [M + 2]+. Relative abundance data were within 1% with the use of either conventional scanning.
RESULTS AND DISCUSSION

In the present study the terminology and mathematical expressions are based on the relevant work of Gurpide et al. (11-13). The percentage of labeled molecules (\( \%LM \)) is defined by Equation 1 where \( d_0 \) is the percentage of unlabeled species and \( d_1 \) is the percentage of monodeuterium-labeled species. Contributions from multideuterated species \( (d_0, d_0, \ldots) \) are ignored.

\[
\%LM = 100 \frac{d_1}{d_0 + d_1}
\]

The \( \%LM \) in Equation 2 is a mean of the percentage of labeled molecules of a urinary metabolite for the number of days \( (t) \) of urine collection, i.e., until the percentage of labeled molecules is zero for the \( t + 1 \) day. The percentage of labeled molecules, \( \%LM^t \), refers to the administered estrogen (\( \%LM^{17\beta} = 88.6\% \)). The mass (milligrams) of administered estradiol-17\( \beta \)-4\( ^2\)H is denoted by \( m \) and is a corrected value which includes only \( d_0 \) and \( d_1 \) species.

Analogous with the equation generally used for the radioactive tracer technique, the production rate \( (P) \) of estradiol \((E_2)\) or estrone, or both, is calculated as follows.

\[
P = \frac{\%LM^t(m_0)}{\%LM^0} = \frac{m_0}{t}
\]

The second term corrects for the mass contributed by the administered estradiol-17\( \beta \) to the apparent daily production rate (first term). Since the mean percentage of labeled molecules for estrone is closely similar to the mean percentage for estradiol-17\( \beta \), the calculated production rate based on either of these values is nearly the same. The dosage (mass) of administered estradiol-17\( \beta \)-4\( ^2\)H, the mean percentage of labeled molecules for estrone, estradiol-17\( \beta \), and 16-epi-estriol, and the production rates for estrone and estradiol-17\( \beta \) are given in Table I.

In recent years the complexities of obtaining true secretion or production rates of estrogens in pregnancy have been widely discussed (11-15). The administration of labeled estradiol-17\( \beta \) into the maternal circulation does not furnish sufficient data for the calculation of the total secretion or production rate of estrogens. Instead, the data so obtained allows only the calculation of the secretion or production rate of estradiol-17\( \beta \) in the pool into which the tracer is introduced. Accordingly, in the present study the production rate of estradiol-17\( \beta \) in the maternal pool, which includes also the amount secreted into the maternal circulation by the placenta, is measured. It is encouraging to note that, despite the many variables and intricacies involved in steroid metabolism

### Table I

**Data collected from study of three healthy pregnant women**

For explanation of terms and method of calculation see "Results and Discussion."

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gestation</th>
<th>Dosage</th>
<th>Estrone</th>
<th>Estradiol-17( \beta )</th>
<th>Production rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>A( ^* )</td>
<td>36-39</td>
<td>14.88</td>
<td>32.55</td>
<td>34.60</td>
<td>2.54</td>
</tr>
<tr>
<td>B( ^* )</td>
<td>35-36</td>
<td>3.48</td>
<td>10.22</td>
<td>13.56</td>
<td>6.67</td>
</tr>
<tr>
<td>C( ^* )</td>
<td>37</td>
<td>3.69</td>
<td>5.24</td>
<td>6.02</td>
<td>14.68</td>
</tr>
</tbody>
</table>

\( ^{\*} \) Mass of administered estradiol-17\( \beta \)-4\( ^2\)H represents only \( d_0 \) and \( d_1 \) species; \( \%LM^t \) for estradiol-17\( \beta \), 88.6.

\( ^{\*} \) Time, 4 days.

\( ^{\*} \) \( \%LM^t \) for 16-epi-estriol, 6.17 (time, 4 days); production rate for 16-epi-estriol, 15.3 mg/24 hours.

### Table II

**Available literature values for production rates of estrone and estradiol-17\( \beta \)**

<table>
<thead>
<tr>
<th>Reference</th>
<th>Gestation time</th>
<th>Production rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Estrone Estradiol-17( \beta )</td>
</tr>
<tr>
<td>Gurpide et al. (12)</td>
<td>17</td>
<td>25, 36, 24, 28</td>
</tr>
<tr>
<td>Fishman et al. (16)</td>
<td>10</td>
<td>7, 14, 14</td>
</tr>
<tr>
<td>Siiteri and MacDonald (17)</td>
<td>14</td>
<td>1.5</td>
</tr>
<tr>
<td>Hobbick and Nilsen (18)</td>
<td>19-33</td>
<td>10-27</td>
</tr>
<tr>
<td>Present study</td>
<td>14</td>
<td>2.54</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>6.67</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>14.08</td>
</tr>
</tbody>
</table>

\( ^{\*} \) Estimated from a bar graph.

\( ^{\*} \) Values obtained from different patients at the same gestation time.

### Table III

**Urinary estrogen excretion following injection of estradiol-17\( \beta \)-4\( ^2\)H**

Corrected for recoveries of 45 to 55\% for estrone, estradiol, and estril. Tritiated estrone-sulfate and estradiol-17\( \beta \)-glucuronide were added to urine prior to extraction, whereas tritiated estril was added after enzymic hydrolysis.

<table>
<thead>
<tr>
<th>Estrogen</th>
<th>Days 1-4</th>
<th>Day 5</th>
<th>Total deuterated, estrogen secreted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrone</td>
<td>149.0</td>
<td>164.2</td>
<td>4.05</td>
</tr>
<tr>
<td>Estradiol-17( \beta )</td>
<td>55.2</td>
<td>70.4</td>
<td>1.88</td>
</tr>
<tr>
<td>Estril</td>
<td>1278.5</td>
<td>797.0</td>
<td>-0.9</td>
</tr>
<tr>
<td>16-Epi-estril</td>
<td>13.3</td>
<td>-0.9</td>
<td>0.081</td>
</tr>
</tbody>
</table>

| Patient A | 364.5 | 219.3 | 149.0 | 4.83 |
| Patient B | 1700.0 | 716.3 | -0.9 | 1.19 |
| 16-Epi-estril | 15.3 | 3.7 | 2.9 | 0.004 |

\( ^{\*} \) No deuterated estril could be detected.

\( ^{\*} \) Not corrected for recovery.

\( ^{\*} \) Insufficient amount for quantitative determination.
during pregnancy, the production rates obtained after the injection of milligram amounts of deuterated estradiol are concordant with those reported using a tracer dose of radioactive steroid (Table II). Furthermore, the mean percentage of labeled molecules (analogous to specific activity) of estrone and estradiol-17β is also closely similar as reported in other studies (12, 16, 18).

Two necessary conditions for the identical specific activities, as suggested previously for radioactive tracer studies, may likewise apply to the present work. There is a rapid interconversion of estrone and estradiol-17β (19), and the rate of transfer of estradiol-17β from the maternal to the fetal pool is negligible in comparison with the rate of metabolism of estradiol-17β in the maternal pool (12). It may further be assumed that any mixture of estrone and estradiol-17β which reaches the maternal pool subsequent to fetal transformation is either in the same relative proportion as that formed in the maternal pool or is further converted by maternal tissues to maintain the same proportionality (16). As a result, the mean percentage of labeled molecules of urinary estrone and estradiol-17β is similar.

The deuterium content of the estriol fractions for all three patients was found to be negligible. These results are as one would anticipate because estriol biogenesis in pregnancy is predominantly independent of estrone and estradiol (20). However, a considerable amount of deuterium was detected in the urinary 16-epi-estriol in two women during early pregnancy (Table I). This result suggests that a portion of the urinary 16-epi-estriol can be derived directly from estradiol-17β in addition to the oxidative metabolism of estriol (21), from other precursors, or from both. Similar results have been previously obtained with the aid of radioactive steroids (18).

Urinary estrogen excretion data are given in Table III for two patients. The total mass of deuterated estrogen excreted is calculated by multiplying the total estrogen excretion by the mean percentage of labeled molecules. Recoveries for estrone and estradiol in the present results were similar to those noted by Hob-kirk and Nilsen (18) after injection of estradiol-17β-4,7-²H to pregnant women. The values also compare favorably with those obtained by Pearlman, Pearlman, and Rakoff (3) who administered massive doses of estrone-6,7-³H acetate to pregnant women. The recovery of 16-epi-estradiol is, however, much lower than the values reported by other investigators (18).

Earlier investigations with deuterium-labeled compounds were hampered because of the paucity of suitable analytical tools for sensitive measurement of deuterium content of various metabolite products. Consequently, deuterium-labeled precursors in large doses (ranging from grams to several hundred milligrams) which might create a nonphysiological state had to be administered for reliable analyses (2, 3). The advent of highly sensitive gas chromatographic detection systems combined with mass spectrometry has made it possible to analyze a minute amount of a compound in biological fluids. Such an analytical system has enabled us to administer a relatively small dose of a compound (estradiol-17β-4,7-²H) labeled with a stable isotope. The results obtained so far suggest that stable isotopes can be used instead of radioactive tracers to obtain comparable information.

Recent work has revealed the existence of complex interrelationships in steroid metabolism of mother, fetus, and placenta. The administration of a single isotope-labeled precursor does not furnish sufficient data to evaluate quantitatively the dynamic relationships among them. Such a situation requires that two or more distinctly labeled compounds be introduced into different pools. The distinction of related precursors could be achieved by the incorporation of one or more stable isotopes (e.g. ²H, ¹³C, ¹⁸O) at a specific site or sites which will result in characteristic molecular or fragment ions, or both, in mass spectrometric analyses. A great deal of information regarding steroid metabolism in pregnancy has been made available through the use of radioactive compounds. By necessity, most of these experiments had to be performed in vivo or by a brief perfusion technique. Conclusions drawn from these experiments have been extrapolated to the situation in vivo. The application of stable isotopes will permit conclusions to be obtained directly from studies under completely natural conditions.

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REFERENCES