OESTRIOL-3-GLUCOSIDURONATE, 
A MAJOR URINARY METABOLITE OF OESTRIOL 
AND OESTRIOL-16(17?)-GLUCOSIDURONATE

By 
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ABSTRACT

Oestriol-16-14C (OE3-16-14C) was administered to a non-pregnant woman 
and oestriol-16-14C-16(17?)-glucosiduronate (OE3-16-14C-16(17?)Gl) to a 
pregnant woman and the urinary oestriol conjugates were isolated and 
identified.

More than 85 per cent of the radioactive material administered to the 
two subjects was excreted in the urine within 96 hours.

In both cases more than 16 per cent of the administered radioactive 
material was isolated and identified as oestriol-16-14C-3-glucosiduronate 
(OE3-16-14C-3Gl) and more than 70 per cent as OE3-16-14C-16(17?)Gl.

It is concluded that oestriol-3-glucosiduronate is a major urinary metab-
olite of both oestriol and oestriol-16(17?)-glucosiduronate.

The fate of circulating OE3 in the human is still incompletely understood.
When OE3-16-14C was injected into a newborn anencephalic, less than 1 per 
cent of the radioactive material recovered from the urine was unconjugated 
OE3. Approximately 50 per cent was present as OE3-16(17?)Gl, more than 16 
per cent as oestriol-3-sulphate (OE3-3S) and at least 25 per cent as oestriol-3-
sulphate,16(17?)-glucosiduronate (OE3-3S,16(17?)Gl) (Diczfalusy et al. 1964).
On the other hand, when labelled OE3 was administered to pregnant women 
in midpregnancy, only very small amounts of OE3-3S and OE3-3S,16(17?)Gl

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were detected in their urine. The bulk of urinary radioactive material was excreted as \( \text{OE}_3 \)-16(17?)\( \text{Gl} \) and as so-called »polar glucosiduronate« (Wilson et al. 1964).

In view of the present identification of \( \text{OE}_3 \)-3\( \text{Gl} \) in extracts of pregnancy urine (Beling 1963), the possibility was considered that the »polar glucosiduronate« of Wilson et al. (1964) might be identical with \( \text{OE}_3 \)-3\( \text{Gl} \). Therefore, in this study an attempt was made to isolate and identify \( \text{OE}_3 \)-3\( \text{Gl} \) from the urine following the administration of labelled \( \text{OE}_3 \) and \( \text{OE}_3 \)-16(17?)\( \text{Gl} \).

**EXPERIMENTAL.**

**Abbreviations.** – The following abbreviations are used throughout this paper: \( \text{CCD} \): countercurrent distribution, \( \text{CPM} \): counts per minute, \( \text{DMSO} \): dimethyl sulfoxide, \( \text{dimsyl-} \text{Na} \): sodium methylsulphinyl carbamion in dimethyl sulfoxide, \( \text{DPM} \): disintegrations per minute, \( K \): partition coefficient, \( m.p.: \) melting point, \( \text{OE}_3 \): oestril (oestratriene-3,16a,17\( \beta \)-triol), \( \text{OE}_3 \)-\( \text{S} \): oestril-3-sulphate, \( \text{OE}_3 \)-16(17?)\( \text{Gl} \): oestril-16(17?)-glucosiduronate, \( \text{OE}_3 \)-3\( \text{S} \),16(17?)\( \text{Gl} \): oestril-3-sulphate,16(17?)-glucosiduronate, \( \text{OE}_3 \)-3\( \text{Gl} \): oestril-3-glucosiduronate, \( \text{OE}_3 \)-3\( \text{Me} \): oestril 3-methyl ether, \( \text{OE}_3 \)-17\( \text{Me} \): oestril 17-methyl ether, \( \text{OE}_3 \)-3,17\( \text{diMe} \): oestril 3,17-dimethyl ether, \( \text{OE}_3 \)-16,17\( \text{diMe} \): oestril 16,17-dimethyl ether, \( \text{OE}_3 \)-3,16,17\( \text{triMe} \): oestril 3,16,17-trimethyl ether, \( \text{OE}_3 \)-3\( \text{Ac} \): oestril 3-acetate, \( \text{OE}_3 \)-3,16\( \text{diAc} \): oestril 3,16-diacetate, S. A.: specific activity, \( \text{TLC} \): thin-layer chromatography.

**Clinical material.** – This study was carried out on two volunteers. One of them, Case No. 1 (16 years of age), was admitted to the hospital in a terminal stage of sarcoma and was injected intravenously with 3 \( \mu \)c of oestril-16-\( ^{14} \text{C} \). The other patient (Case No. 2) was 23 years of age and was admitted to the hospital for interruption of pregnancy in the 19th week of gestation. Permission for termination of gestation was granted upon request of the patient by the Royal Medical Board. This patient was given a continuous intravenous infusion of 1 \( \mu \)c of oestril-16-\( ^{14} \text{C} \)-16(17?)-glucosiduronate over a period of 310 minutes.

**Material administered.** – Chromatographically pure \( \text{OE}_3 \)-16-\( ^{14} \text{C} \) with a S. A. of 22 \( \mu \)c per mg\(^2 \) was further purified by TLC using system A of Lisboa and Dicefalusy (1962).

\( \text{OE}_3 \)-16-\( ^{14} \text{C} \)-16(17?)\( \text{Gl} \) obtained from the urine of four volunteers injected with \( \text{OE}_3 \)-16-\( ^{14} \text{C} \)\(^3 \) was purified as follows: the urine was submitted portionwise to gel filtration on Sephadex G-25 (Beling 1963) and the peak II material was subjected in 6 aliquots to CCD in system No. 1 (average \( K = 0.86 \)). The \( \text{OE}_3 \)-16(17?)\( \text{Gl} \)-like material was pooled and submitted to CCD in system No. 2 (\( K = 0.75 \)) and then No. 3 (\( K = 1.4 \)). Further purification was achieved by an O'Keefe (1949) distribution in system No. 4 using 1250 transfers\(^4 \). The \( \text{OE}_3 \)-16-\( ^{14} \text{C} \)-16(17?)\( \text{Gl} \)-like material was then submitted to CCD in system No. 1 (\( K = 1.0 \)) and the radiochemically homogeneous material was freed from solvent residues by gel filtration. The \( \text{OE}_3 \)-16-\( ^{14} \text{C} \)-16(17?)\( \text{Gl} \) with a S. A.

2. Obtained through the courtesy of Dr. M. Levitz, New York City, N. Y., U. S. A.
3. Reported in a detailed previous communication (Wilson et al. 1964).
4. Distributions involving more than 36 transfers were carried out in an automatic steady state distribution machine purchased from Quickfit and Quartz, Ltd., Stone, Staffordshire, England.
of 4.4 \( \mu c \) per mg of oestriol was kept in 70 per cent (v/v) ethanol for 4 days prior to use.

Administration of radioactive material and urine collection. – Following the intravenous injection of 3 \( \mu c \) of OE\textsubscript{3}-16\textsuperscript{14}C, four 24-hour urine specimens were collected from Case No. 1. In Case No. 2, approximately 1 \( \mu c \) of oestriol-16\textsuperscript{14}C-16(17\textsuperscript{?})-glucosiduronate was administered over 310 minutes as a continuous intravenous infusion in saline at a rate of 3.2 ml/min\textsuperscript{8}. Hourly urine samples were collected by an indwelling catheter during the first 7 hours, followed by the collection of one 5-hour and two 6-hour specimens.

Reference standard preparations. – OE\textsubscript{3}-17Me and OE\textsubscript{3}-16,17diMe have been obtained through the courtesy of Ing. K.-E. Lundvall, AB Leo, Hälsingborg, Sweden. OE\textsubscript{3}-17Me was prepared from OE\textsubscript{3}-3,16diAc by the method of Neeman and Hashimoto (1962), m. p. 188–190\textdegree \textsuperscript{uncorr} \textsuperscript{9} (reported: 192–192.5\textdegree \textsuperscript{corr}). OE\textsubscript{3}-16,17diMe was prepared according to the method of Neeman et al. (1959) from OE\textsubscript{3}-3Ac\textsuperscript{a} and was recrystallized from methanol: m. p. 205–208\textdegree \textsuperscript{uncorr}. Calculated CH\textsubscript{2}O for C\textsubscript{2}H\textsubscript{23}O\textsubscript{3}: 19.6, found: 19.0. OE\textsubscript{3}-3,17diMe was prepared from OE\textsubscript{3}-17Me by methylation according to Brown (1955). As evidenced by CCD studies, no 16-methoxy derivative was formed.

OE\textsubscript{3}-3,16,17triMe was prepared\textsuperscript{a} by the method of Sjöberg & Sjöberg (1965) as follows: 500 mg (1.7 mmoles) of OE\textsubscript{3} were dissolved in 5.0 ml of anhydrous DMSO together with traces of triphenylmethane as an indicator. Upon the addition of 3.50 ml (5.2 mmoles) of freshly prepared 1.48 \( n \) dimethyl-Na, the red colour of the triphenylmethane anion appeared, indicating that the three hydroxylic protons of OE\textsubscript{3} had been removed. After the addition of another 0.5 ml of dimethyl-Na, 950 mg (7.5 mmoles) of dimethyl sulphate were added dropwise under cooling. The solution was allowed to stand for 5 minutes at 20\textdegree. Dichloromethane and water were added under cooling. The dichloromethane extracts were washed 4 times with water and were dried over MgSO\textsubscript{4}. Following evaporation of the solvent, a crystalline residue of 580 mg was obtained. After recrystallisation from 90 per cent methanol (v/v), the compound melted at 88–90\textdegree, resolidified and melted again at 111–113\textdegree \textsuperscript{corr}. Yield: 480 mg (1.5 mmoles, 88\%). After four recrystallisations, m. p. 111–113\textdegree \textsuperscript{corr}. Calculated for C\textsubscript{2}H\textsubscript{23}O\textsubscript{3} (330.5): C 76.3; H 9.15. Found C 76.4; H 9.19. The infrared spectrum showed no bands between 3700 and 3200 cm\textsuperscript{-1}, indicating the absence of hydroxyl groups. The nuclear magnetic resonance spectrum, obtained at 60 Mc/s with a Varian A60 spectrometer, showed three equally strong signals at \( \delta = 3.72, 3.46 \) and 3.32 ppm, indicating one aromatic and two aliphatic methoxy groups.

Hydrolysis of completely methylated oestriol conjugates was carried out by refluxing the material in 4 \( n \) HCl in 50 per cent (v/v) ethanol for 4 hours.

Enzymic hydrolysis of oestriol glucosiduronates was carried out by the use of a bacterial \( \beta \)-glucuronidase preparation\textsuperscript{7} using the conditions described previously (Dick-falusy et al. 1961).

Koher reaction for OE\textsubscript{3} and for its various derivatives was carried out by the method of Brown (1955), using his OE\textsubscript{3}-reagent. In TLC the specifications of Lisboa & Dick-falusy (1962) were followed.

Countercurrent distribution. – Unless otherwise stated, 24-transfer countercurrent distributions were carried out. The following systems were used:

5. By the use of a Model 2 pump purchased from Process & Instruments, 15 Stone Ave., Brooklyn, N. Y., U. S. A.
6. m. p. 181–183\textdegree \textsuperscript{uncorr}.
7. Purchased from Sigma Chemical Co., St. Louis, Mo., U. S. A.
No. 1: n-Butanol, ethyl acetate, 0.2 \( \times \) NH\(_4\)OH (1:1:2)
2.: n-Butanol, sec. butanol, 2 \( \times \) NH\(_4\)OH (1:1:2)
3.: Ethyl acetate, 0.1 \( \times \) HCl (1:1)
4.: n-Butanol, 2 \( \times \) NH\(_4\)OH (1:1)
5.: n-Butanol, tert. butanol, 2 \( \times \) NH\(_4\)OH (13:7:20)
6.: n-Butanol, n-hexane, 0.1 \( \times \) NaOH (3:1:4)
7.: sec. Butanol, 2 \( \times \) NH\(_4\)OH (1:1)
8.: n-Butanol, water /pH 2.5/ (1:1)
9.: sec. Butanol, water /pH 7.0/ (1:1)
10.: Benzene, water (1:1)
11.: Methanol, water, carbon tetrachloride (2:3:5)
12.: Benzene, n-hexane, methanol, water (3:7:5:5)
13.: n-Hexane, methanol, water (10:7:3)
14.: n-Hexane, methanol, water (10:9:1).

Measurement of radioactivity. – A Model 3000 Tri-Carb liquid scintillation spectrometer system and the scintillation liquid described by Bray (1960) were used. One half ml aliquots of untreated urine or of aqueous samples were mixed with 15 ml of scintillation liquid. With 0.5 ml of water in 15 ml of scintillation liquid, the efficiency for \(^3\)H was 15.5 per cent and that for \(^14\)C was 43 per cent. Forty-four per cent of the \(^14\)C counts appeared in the \(^4\)H channel, and 0.1 \%/o of the \(^3\)H counts in the \(^14\)C channel. With the settings employed, the average background in the \(^4\)H channel was 30 CPM, and in the \(^14\)C channel 18 CPM. Tritium- and \(^14\)C efficiencies as well as the contamination of the \(^4\)H channel with \(^14\)C counts were monitored in all instances by the subsequent addition of \(^4\)H- and \(^14\)C-labelled toluene. Throughout this paper, all results of radioactivity measurements are expressed in DPM.

Analytical method

In Case No. 1, the four 24-hour urine specimens were submitted to gel filtration (Beling 1963). Virtually all urinary radioactive material was eluted as peak I and peak II material. The peak I material was further purified as described below under the identification of OE\(_3\)-3GI. The peak II material was submitted to CCD in system No. 1 (K = 1.0) and was further characterized as described under the identification of OE\(_3\)-16[17?]Gl.

In Case No. 2, the 10 urine specimens collected during the 24 hours of the experiment were submitted separately to CCD in system No. 1 and the OE\(_3\)-3GI-like material was separated from the OE\(_3\)-16[17?]Gl-like material. The OE\(_3\)-3GI-like material was then pooled for the purpose of identification.

RESULTS

Urinary excretion of OE\(_3\)-16-\(^14\)C-3GI following the administration of OE\(_3\)-16-\(^14\)C and OE\(_3\)-16-\(^14\)C-16[17?]Gl. – The urinary excretion of OE\(_3\)-16-\(^14\)C-3GI is compared with that of OE\(_3\)-16-\(^14\)C-16[17?]Gl in Tables 1 (Case No. 1) and 2 (Case No. 2).

The data of Table 1 indicate that more than 85 per cent of the radioactive material administered as OE\(_3\)-16-\(^14\)C was excreted within 96 hours. During the first 24 hours, 11 per cent of the urinary radioactivity was excreted as OE\(_3\)-
Radioactive material recovered from the urine during four consecutive days following a single intravenous injection of 3.0 μC of oestriol-16-¹⁴C to a non-pregnant volunteer (Case No. 1). OE₃-16(17?)GI indicates oestriol-16(17?)-glucosiduronate and OE₃-3GI stands for oestriol-3-glucosiduronate.

### Table 1.

<table>
<thead>
<tr>
<th>Period of urine collection</th>
<th>Radioactive material excreted (DPM x 10⁶)</th>
<th>Percentage of administered dose</th>
<th>OE₃-16(17?)GI (DPM x 10⁶)</th>
<th>OE₃-3GI (DPM x 10⁶)</th>
<th>OE₃-3GI as percentage of excreted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>4559</td>
<td>68.4</td>
<td>4070</td>
<td>489</td>
<td>10.7</td>
</tr>
<tr>
<td>Day 2</td>
<td>720</td>
<td>10.8</td>
<td>300</td>
<td>420</td>
<td>58.3</td>
</tr>
<tr>
<td>Day 3</td>
<td>334</td>
<td>5.0</td>
<td>188</td>
<td>146</td>
<td>43.7</td>
</tr>
<tr>
<td>Day 4</td>
<td>91</td>
<td>1.4</td>
<td>51</td>
<td>40</td>
<td>44.0</td>
</tr>
<tr>
<td>Total:</td>
<td><strong>5704</strong></td>
<td>85.6</td>
<td><strong>4609</strong></td>
<td><strong>1095</strong></td>
<td><strong>19.2</strong></td>
</tr>
</tbody>
</table>

Recovery of radioactive material from the urine as oestriol-16(17?)-glucosiduronate (OE₃-16(17?)GI), oestriol-3-glucosiduronate (OE₃-3GI) and unconjugated oestriol (OE₃) following the intravenous infusion of oestriol-16-¹⁴C-16(17?)-glucosiduronate (Case No. 2). Infusion period: 310 min.

### Table 2.

<table>
<thead>
<tr>
<th>Hours of urine collection</th>
<th>Amount excreted (DPM x 10⁻³)</th>
<th>Percentage distribution of conjugated and unconjugated oestriol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OE₃-16(17?)GI</td>
<td>OE₃-3GI</td>
</tr>
<tr>
<td>1</td>
<td>31</td>
<td>93.5</td>
</tr>
<tr>
<td>2</td>
<td>43</td>
<td>92.4</td>
</tr>
<tr>
<td>3</td>
<td>72</td>
<td>94.9</td>
</tr>
<tr>
<td>4</td>
<td>201</td>
<td>93.0</td>
</tr>
<tr>
<td>5</td>
<td>364</td>
<td>92.8</td>
</tr>
<tr>
<td>6</td>
<td>567</td>
<td>91.8</td>
</tr>
<tr>
<td>7</td>
<td>24</td>
<td>77.8</td>
</tr>
<tr>
<td>8-12</td>
<td>197</td>
<td>51.0</td>
</tr>
<tr>
<td>13-18</td>
<td>250</td>
<td>65.6</td>
</tr>
<tr>
<td>19-24</td>
<td>156</td>
<td>46.4</td>
</tr>
<tr>
<td>Total in 24 hours:</td>
<td>1905b)</td>
<td>80.6</td>
</tr>
</tbody>
</table>

²) These values were calculated from the results of countercurrent distributions rather than ether-water partitions. Therefore it can not be stated how much of this material was excreted originally as unconjugated OE₃ and how much was formed artifactually during the procedure employed.

b) Represents 89.7 per cent of the material infused.
3Gl-like material. However, from the second 24-hour specimen approximately equal amounts of OE₃-3Gl- and OE₃-16(17?)Gl-like material were excreted. These data are in close agreement with those reported by Wilson et al. (1964) and indicate that the pattern of excretion is similar in pregnant and non-pregnant individuals.

The data of Table 2 indicate that more than 89 per cent of the radioactive material infused as OE₃-16-¹⁴C-16(17?)Gl was excreted within 24 hours. During the infusion period, only some 6 per cent of the urinary radioactivity was excreted as OE₃-3Gl-like material. However, following discontinuation of the infusion, OE₃-3Gl-like material accounted for at least 40 per cent of the total urinary excretion.

In both cases studied, the OE₃-3Gl-like material amounted to approximately 19 per cent of the total radioactive material excreted (i.e. more than 16 per cent of the administered dose).

Identification of the urinary oestriol conjugates

Oestriol-16-¹⁴C-16(17?)-glucosiduronate. – Aliquots of the pooled OE₃-16(17?)Gl-like radioactive material from both cases were mixed separately with authentic OE₃-16(17?)Gl* and were subjected to a series of CCD in systems No. 3 (K = 1.4), No. 4 (K = 0.59) and No. 5 (K = 1.0), and following methylation according to Brown in system No. 6. As expected from previous studies (Troen et al. 1961; Diczfalussy et al. 1961), both the authentic carrier and the labelled OE₃-16(17?)Gl-like material yielded two peaks (K = 0.64 and K = 3.0).

The OE₃-16-¹⁴C-16(17?)Gl-like material obtained from the urine of Case No. 1 was also submitted to a 1000-transfer CCD in system No. 1 (K = 1.05). No radioactive material whatsoever could be dissociated from the carrier in any of the above distributions.

More than 94 per cent of the OE₃-16-¹⁴C-16(17?)Gl-like material obtained from both cases was hydrolysed by β-glucuronidase in the absence and less than 14 per cent in the presence of 10 mg/ml of D-saccharic acid lactone.

Oestriol-16-¹⁴C-3-glucosiduronate. – The OE₃-3Gl-like radioactive material collected from all urine specimens from Case No. 1 was pooled and submitted to an O’Keefe (1949) distribution in system No. 7, using 1600 transfers. The radioactive material exhibiting a K-value of 0.4 or more was collected and submitted to a 36-transfer CCD in system No. 1 (K = 0.05), followed by three subsequent CCD in systems No. 7 (K = 0.45), No. 8 (K = 5.3) and No. 9

8. The »polar material« of Wilson et al. (1964) exhibits the same partition characteristics as OE₃-3Gl in all systems studied.

9. Oestriol-16(17?)-glucosiduronate was a generous gift of Professor W. Allen, St. Louis, Mo., U.S.A.
The last four distributions indicated the presence of a single compound. This material was used for further identification.

The OE₃-3Gl-like material obtained from the 10 urine specimens from Case No. 2 was combined and submitted to a 600-transfer CCD in system No. 1 (K = 0.09). Following the removal of solvent residues by gel filtration and an additional CCD in system No. 1 (K = 0.11), this material was used for further identification.

An aliquot of the labelled OE₃-3Gl-like material from both cases was hydrolysed with β-glucuronidase, as shown in Table 3.

<table>
<thead>
<tr>
<th>Concentration of D-saccharic acid lactone (mg per ml)</th>
<th>Percentage hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OE₃-16(17?)Gl</td>
</tr>
<tr>
<td>0</td>
<td>97.3</td>
</tr>
<tr>
<td>10</td>
<td>1.5</td>
</tr>
<tr>
<td>20</td>
<td>0.2</td>
</tr>
</tbody>
</table>

The data of Table 3 indicate that the OE₃-3Gl-like material was as readily hydrolysed by the enzyme as OE₃-16(17?)Gl and that this hydrolysis was completely inhibited by D-saccharic acid lactone, although more inhibitor was needed to achieve a complete inhibition of the hydrolysis of OE₃-3Gl than in the case of OE₃-16(17?)Gl. This is in agreement with previous data on the hydrolysis of oestrone glucosiduronate (Diczfalusy et al. 1962).

Since from this stage the identification of the OE₃-3Gl-like radioactive material obtained from Cases No. 1 and 2 was carried out in a parallel and identical fashion, the procedure will only be described in detail for the material obtained from Case No. 1.

The method of identification is based on a modification of the principle of Neeman & Hashimoto (1962). By the use of the present forced methylation technique – described in detail in the experimental part in connection with the preparation of OE₃-3,16,17-triMe – all hydroxyl groups of all oestriol conjugates will be methylated, except those bound as glucosiduronates or sulphates. The sulphate or glucosiduronate moieties are subsequently removed by hot acid hydrolysis, and the resulting oestriol methyl ethers are identified. Finally,
a repeated methylation, carried out by the method of Brown (1955) will only result in the methylation of the aromatic hydroxyl group (e.g. Brown 1955: Hertelendy & Common 1963). The differences in the partition characteristics of the various methyl ethers are so marked that they can be completely separated in a 24-transfer CCD, as shown in Table 4.

Table 4.

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>OE₃</th>
<th>OE₃-17Me</th>
<th>OE₃-3Me</th>
<th>OE₃-16,17diMe</th>
<th>OE₃-3,17diMe</th>
<th>OE₃-3,16,17triMe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene, water (1:1)</td>
<td>0.19</td>
<td>15.7</td>
<td>24.0</td>
<td>a)</td>
<td>a)</td>
<td>a)</td>
</tr>
<tr>
<td>Methanol, water, carbon tetrachloride (2:3:5)</td>
<td>13.3</td>
<td>2.7</td>
<td>0.77</td>
<td>0.03</td>
<td>0.01</td>
<td>b)</td>
</tr>
<tr>
<td>Benzene, n-hexane, methanol, water (3:7:5:5)</td>
<td>&lt;0.01</td>
<td>0.05</td>
<td>0.15</td>
<td>2.85</td>
<td>6.1</td>
<td>15.7</td>
</tr>
<tr>
<td>n-Hexane, methanol, water (10:7:3)</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.01</td>
<td>0.09</td>
<td>0.23</td>
<td>13.3</td>
</tr>
<tr>
<td>n-Hexane, methanol, water (10:9:1)</td>
<td>b)</td>
<td>b)</td>
<td>b)</td>
<td>0.04</td>
<td>0.11</td>
<td>2.8</td>
</tr>
</tbody>
</table>

a) K-values not estimated, but certainly greater than 24.
b) K-values not estimated, but certainly smaller than 0.01.

The K-values shown in Table 4 indicate incomplete separation of OE₃-3,17diMe from OE₃-16,17diMe, at least in a 24-transfer distribution. Complete separation can be obtained either by an increase of the number of transfers, or following repeated methylation according to Brown.

To explore the validity of the present procedure, a mixture of tracer amounts of OE₃-16-¹⁴C₂ and of OE₃-15-³H-³S₂ was submitted to CCD in system No. 14 following forced methylation and hot acid hydrolysis. The ¹⁴C-labelled material – with an over-all yield of 58 per cent – exhibited the same K-value as the simultaneously distributed authentic OE₃-3,16,17triMe (K = 2.5), whereas the ³H-labelled material had a K-value of 0.04, corresponding to that of OE₃-16,17diMe. Also when distributed in system No. 12, the ³H-labelled material behaved as OE₃-16,17diMe (K = 2.6), whereas following methylation according
to Brown its distribution in system No. 14 agreed with that of authentic OE₃-3,16,17triMe (K = 2.7). The over-all yield was 52 per cent. It follows from these data that the technique of forced methylation described in this paper does not hydrolyse OE₃-3S.

In the next experiment an aliquot of the purified urinary OE₃-16-¹⁴C-3G₁-like material was mixed with OE₃-15-³H-16(17?)G₁. Following forced methylation and hot acid hydrolysis, CCD in system No. 12 indicated the presence of a ¹⁴C-labelled compound with a K-value of 3.0 (OE₃-16,17diMe: K = 2.8), and of a ³H-labelled product with K = 6.1 (OE₃-3,17diMe: K = 6.1). In system No. 13 the ¹⁴C-labelled material had a K-value of 0.09 (OE₃-16,17diMe: K = 0.09), whereas the distribution of the ³H-labelled material agreed with that of the simultaneously distributed authentic OE₃-3,17diMe (K = 0.23). Following methylation according to Brown and addition of carrier OE₃-3,16,17-triMe, the mixture was submitted to CCD in system No. 14. The distribution of the ³H-labelled material agreed with that of carrier OE₃-3,17diMe (K = 0.12) and the distribution of the ¹⁴C-labelled material with that of carrier OE₃-3,16,17triMe (K = 2.7). Sixty-seven per cent of the ³H-labelled and 44 per cent of the ¹⁴C-labelled starting material was recovered in the form of OE₃-3,17diMe and OE₃-3,16,17triMe, respectively.

Another aliquot of the ¹⁴C-labelled OE₃-3G₁-like material was mixed with OE₃-15-³H-3S² and carried through the same procedure. When submitted to CCD in system No. 12, both the ¹⁴C- and ³H-labelled material gave an identical distribution curve (K = 2.8) with a constant isotopic ratio (³H/¹⁴C = 33). Following methylation according to Brown, the labelled OE₃-3,16,17triMe formed was mixed with authentic standard and recrystallized to constant specific activity, as shown in Table 5. The isotopic ratio remained unchanged.

### Table 5.

<table>
<thead>
<tr>
<th>Crystallisation</th>
<th>³H</th>
<th>¹⁴C</th>
<th>³H/¹⁴C</th>
<th>³H</th>
<th>¹⁴C</th>
<th>³H/¹⁴C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Ethanol</td>
<td>3130</td>
<td>94</td>
<td>33</td>
<td>3690</td>
<td>111</td>
<td>33</td>
</tr>
<tr>
<td>2. Methanol</td>
<td>3120</td>
<td>94</td>
<td>33</td>
<td>3525</td>
<td>107</td>
<td>33</td>
</tr>
<tr>
<td>3. Ethanol</td>
<td>2980</td>
<td>90</td>
<td>33</td>
<td>3069</td>
<td>91</td>
<td>34</td>
</tr>
<tr>
<td>4. Methanol</td>
<td>2970</td>
<td>93</td>
<td>32</td>
<td>3100</td>
<td>83</td>
<td>37</td>
</tr>
</tbody>
</table>

Calculated: ³H: 3180, ¹⁴C: 95, ³H/¹⁴C: 33.

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during methylation and recrystallisation. The over-all yield of $^{14}$C-labelled OE$_3$-3,16,17triMe was 67 per cent and that of the $^3$H-labelled product was 86 per cent.

**DISCUSSION**

The end-products which can be obtained from various oestriol conjugates by the use of the present method of identification are summarized in Table 6.

It can be seen that only OE$_3$-3Gl and OE$_3$-3S will yield OE$_3$-16,17diMe in the first stage of the identification and OE$_3$-3,16,17triMe in the second stage. The polarities of these two conjugates are so markedly different (e.g. Menini & Diczfalussy 1961; Wilson et al. 1964) that they are easily separated by simple partition methods.

The data of this paper also indicate that by the use of the method described, tracer amounts of oestriol conjugates can be identified with a satisfactory yield. Thus in the last experiment described, 67 per cent of the urinary OE$_3$-3Gl-like radioactive material was isolated in form of crystalline OE$_3$-3,16,17-triMe. Crystallisation of the corresponding material obtained from OE$_3$-3Gl from Case No. 2 gave an over-all yield of 52 per cent.

The present isolation and identification of OE$_3$-3Gl confirms the previous identification of this compound in pregnancy urine (Beling 1963) and indicates that this compound is an important urinary metabolite of both OE$_3$ and OE$_3$-16(17?)Gl. More than 16 per cent of the radioactive material administered either as OE$_3$ or as OE$_3$-16(17?)Gl was recovered from the urine as OE$_3$-3Gl.

A comparison of the partition characteristics of OE$_3$-3Gl with those of conjugate C of Troen et al. (1961), or with those of the polar glucosiduronate found following perfusion of human intestines with OE$_3$ (Diczfalusy et al. 1961), or following the administration of OE$_3$ to pregnant women (Wilson et al. 1964) suggests that all these previously detected compounds were identical with OE$_3$-3Gl, rather than with a di- or triglucosiduronate of OE$_3$.

The results of the present study also indicate that more than 85 per cent of the radioactive material administered as OE$_3$ or OE$_3$-16(17?)Gl is excreted in the urine within 4 days. This is in agreement with the data reported by Sandberg & Slaunwhite (1965). However, our data contradict the conclusion of these authors that OE$_3$-16(17?)Gl was not split significantly in the body, since more than 16 per cent of the administered OE$_3$-16(17?)Gl was shown to be further metabolised into OE$_3$-3Gl.

**ACKNOWLEDGEMENTS**

We are indebted to Professor W. Allen, St. Louis, Mo., U. S. A., for a generous gift of oestriol-16(17?)-glucosiduronate, to Dr. M. Levitz, New York City, N. Y., U. S. A.,

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Table 6.
End-products formed from oestriol and oestriol conjugates following 1) forced methylation and hot acid hydrolysis and 2) subsequent methylation according to Brown (1955).

<table>
<thead>
<tr>
<th>Starting material</th>
<th>Expected end-product following</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1) Forced methylation and hot acid hydrolysis</td>
</tr>
<tr>
<td>Oestriol</td>
<td>Oestriol 3,16,17-trimethyl ether</td>
</tr>
<tr>
<td>Oestriol-3-sulphate</td>
<td>Oestriol 16,17-dimethyl ether</td>
</tr>
<tr>
<td>Oestriol-3-glucosiduronate</td>
<td>Oestriol 3,17-dimethyl ether</td>
</tr>
<tr>
<td>Oestriol-16-glucosiduronate</td>
<td>Oestriol 3,16-dimethyl ether</td>
</tr>
<tr>
<td>Oestriol-17-glucosiduronate</td>
<td>Oestriol 17-methyl ether</td>
</tr>
<tr>
<td>Oestriol-3,16-diglucosiduronate</td>
<td>Oestriol 3,17-dimethyl ether</td>
</tr>
<tr>
<td>Oestriol-3,17-diglucosiduronate</td>
<td>Oestriol 3-methyl ether</td>
</tr>
<tr>
<td>Oestriol-16,17-glucosiduronate</td>
<td>Oestriol 17-methyl ether</td>
</tr>
<tr>
<td>Oestriol-3-sulphate-16-glucosiduronate</td>
<td>Oestriol 16-methyl ether</td>
</tr>
<tr>
<td>Oestriol-5-sulphate-17-glucosiduronate</td>
<td>Oestriol</td>
</tr>
<tr>
<td>Oestriol-3,16,17-triglucosiduronate</td>
<td>Oestriol</td>
</tr>
<tr>
<td>Oestriol-3-sulphate-16,17-diglucosiduronate</td>
<td>Oestriol</td>
</tr>
<tr>
<td></td>
<td>Oestriol</td>
</tr>
</tbody>
</table>
for a generous supply of oestriol-16-14C, oestriol-15-3H-3-sulphate and oestriol-15-3H-16(17?)-glucosiduronate, and to Ing. K.-E. Lundvall, AB Leo, Hälsingborg, Sweden, for providing us with oestriol 17-methyl ether and oestriol 16,17-dimethyl ether. For the NMR-spectrum we are indebted to Mr. K. I. Dahlqvist, Stockholm. The elementary analysis of oestriol trimethyl ether was carried out at the Centrala Analyslaboratoriet, Uppsala, Sweden.

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