A NEW PATHWAY OF OESTRIOL BIOSYNTHESIS IN PREGNANCY?

By

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ABSTRACT

Incubation of \([^{14}C]\)pregnenolone with sliced adrenal tissue from a newborn anencephalic infant yielded \([^{14}C]16\alpha\)-hydroxy-dehydroepiandrosterone \((16\alpha\text{-OH-D})\). When \([^3\text{H}]\text{dehydroepiandrosterone (D)}\) was incubated with this tissue, \([^3\text{H}]16\alpha\text{-OH-D}\) could not be detected. Incubation of \([^3\text{H}]\text{pregnenolone and }[^{14}C]\text{D together in equimolar quantities yielded }16\alpha\text{-OH-D containing }^3\text{H but not }^{14}\text{C. This evidence suggests that }16\alpha\text{-OH-D can be formed }in\ vitro\ by the adrenal of the newborn anencephalic infant from a C\text{21} steroid by a pathway which avoids D. }16\alpha\text{-OH-D is an efficient precursor of oestriol in late pregnancy. The pathway demonstrated }in\ vitro\ could therefore provide another source of oestriol precursor from foetal tissues, similar to that which Kirschner }et\ al.\ (1966)\ suggested may operate in maternal tissues.

16\alpha\text{-Hydroxy-dehydroepiandrosterone and its sulphate (16\alpha\text{-OH-DS}) have been recognized as major precursors of oestriol found in maternal urine during the last trimester of pregnancy. The concentration of 16\alpha\text{-OH-D and 16\alpha\text{-OH-DS in cord blood from anencephalic infants is much lower than in cord blood from normal infants (Easterling et al. 1966) and oestriol excretion is diminished in pregnancy with an anencephalic foetus (Frandsen & Stakemann 1961). These compounds or their immediate precursors, therefore, originate mainly from the foetal zone of the foetal adrenal, which is atrophic in the anencephalic foetus. It is generally considered that the foetal adrenal secretes D and its sulphate...}
(DS) which are hydroxylated by the foetal liver (Heinrichs et al. 1966) to provide 16α-OH-D and 16α-OH-DS for oestriol biosynthesis.

Other sources of precursors of urinary oestriol must, however, be considered. MacDonald & Siteri (1965) showed that androgens from the maternal adrenal could contribute to oestrogen biosynthesis. Furthermore, Kirschner et al. (1966) suggested that alternative pathways of oestriol synthesis may operate, possibly involving 16α-hydroxy-C₂₁ steroids. Their conclusion was derived from a comparison of the specific activities of urinary oestrone, 17β-oestradiol and oestriol after injection of [3H]DS into the maternal circulation. Their findings have been substantiated by Young Lai & Solomon (1968).

Shahwan et al. (1968) reported that adrenal tissue from newborn anencephalic infants converted [14C]pregnenolone to [14C]16α-OH-D in vitro. It was not possible from this data to exclude a pathway which utilized D as an intermediate.

We now report the isolation of 16α-OH-D containing ³H, but not ¹⁴C, after incubation of [³H]pregnenolone and [¹⁴C]D with adrenal tissue from a newborn anencephalic infant. In addition, the failure of adrenal tissue from other anencephalic infants to convert [³H]D to [³H]16α-OH-D is described. These results provide strong evidence for the biosynthesis of 16α-OH-D in the definitive zone of the foetal adrenal, at least in vitro, by a pathway which avoids D.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals, reagents, solvents and supporting phases for chromatography were obtained from the same sources as previously described (Shahwan et al. 1968).

Steroids

[7α-³H]Pregnenolone (2500 mc/mmole), [4-¹⁴C]pregnenolone (24.0 mc/mmole), [4-¹⁴C]D (27.5 mc/mmole) and [7α-³H]D (3400 mc/mmole) were obtained from The Radiochemical Centre, Amsersham. The specific activity was adjusted when necessary by addition of purified non-radioactive steroid. Each steroid gave a single radioactive peak with mobility identical to that of an authentic sample on chromatography in system III (see below), and all were used without further purification. 16α-OH-D was provided by Dr. S. Solomon, Montreal, 16α-hydroxy-oestrone by the M.R.C. Steroid Reference Collection. Pregnenolone, D and oestriol were obtained from commercial suppliers.

Cofactors

Adenosine triphosphate (ATP), nicotinamide-adenine dinucleotide phosphate, reduced form (NADPH) and nicotinamide-adenine dinucleotide (NAD) were obtained from Sigma London Chemical Co. Ltd., London, S. W. 6.
Measurement of radioactivity

³H and ¹⁴C were determined in fractions of eluate and located on paper chromatograms as described by Shahwan et al. (1968).

Determination of oestriol and oestrone

Oestriol and oestrone eluted from partition columns were determined by fluorimetry with sulphuric acid as described by Oakey et al. (1963).

Detection of steroids on paper chromatograms

Δ⁵-Δ⁶-Hydroxy steroids were detected with phosphomolybdic acid, and oestrogens with potassium ferricyanide-ferric chloride reagent as described by Bush (1951).

Paper chromatography

The following solvent systems were used at 20° C on Whatman no. 1 paper:

- system I: light petroleum (b. p. 40–60° C), toluene, methanol, water (5:5:7:3 by vol.),
- system II: benzene, methanol, water (10:7:3 by vol.),
- system III: light petroleum (b. p. 80–100° C), methanol, water (10:8:2 by vol.),
- system IV: light petroleum (b. p. 80–100° C), methanol, water (100:96:4 by vol.).

Column partition chromatography

Celite partition columns were prepared in glass tubes 17 cm long and 1 cm internal diameter. The solvent systems used were:

- system V: ethylene dichloride, methanol, water (10:7:3 by vol., Bauld 1955),

Acetylation, reduction and hydrolysis

Steroids were acetylated with acetic anhydride and reduced with NaBH₄ as described by Bush (1961). Steroid acetates were hydrolyzed with Na₂CO₃ (Carballeira & Venning 1964).

Tissue

Adrenal glands were obtained from 3 newborn anencephalic infants (cases A, B and C) within 0.5 h of death (Fig. 1). An earlier investigation with tissue from case B has been published (Shahwan et al. 1968). The weights of the pairs of adrenal glands in the 3 cases were 0.74 g, 0.80 g and 0.58 g respectively. The definitive and atrophic foetal zones were not separated.

Incubation and extraction of steroids

All incubations were carried out in Krebs-Ringer phosphate buffer (5 ml, pH 7.3) for 5 h with air as the gas phase at 37° C with shaking. The amount of sliced adrenal tissue and substrate used in each incubation is shown in Table 1. After the incubation, acetone (40 ml) was added together with non-radioactive carrier steroids including D
Section of adrenal of anencephalic infant (a) and of normal infant (b).
Haemotoxylin and eosin (× 200).

and 16α-OH-D (100 μg of each). The residue from an ether extract of the incubation medium was partitioned between 70% aqueous methanol and hexane. The aqueous methanol solution was adjusted to 50% and extracted with ether. The ether extract was washed with NaOH to remove phenolic steroids. The residue after evaporation of the ether was the »neutral steroid« fraction.

Control experiment
Adrenal tissue from a stillborn anencephalic infant was boiled and incubated
Table 1.
Weights of adrenal tissue and radioactive steroids incubated.

<table>
<thead>
<tr>
<th>Case</th>
<th>Incubation no.</th>
<th>Tissue incubated mg</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>200</td>
<td>[4-14C]Pregnenolone (1.9 µc, 25 µg)</td>
</tr>
<tr>
<td>A</td>
<td>2</td>
<td>200</td>
<td>[7α-3H]D (10 µc, 24 µg)</td>
</tr>
<tr>
<td>B</td>
<td>3</td>
<td>185</td>
<td>[4-14C]Pregnenolone (1.9 µc, 25 µg)</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>185</td>
<td>[7α-3H]D (6.4 µc, 24 µg)</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>145</td>
<td>[7α-3H]Pregnenolone (19 µc, 24 µg) and [4-14C]D (0.77 µc, 22 µg)</td>
</tr>
<tr>
<td>D</td>
<td>6</td>
<td>400</td>
<td>[4-14C]Pregnenolone (0.4 µc, 5.27 µg)</td>
</tr>
<tr>
<td>D</td>
<td>7</td>
<td>370</td>
<td>[7α-3H]D (boiled) (10 µc, 24 µg)</td>
</tr>
</tbody>
</table>

separately with [14C]pregnenolone and [3H]D. A »neutral steroid« fraction was prepared as described above.

Conversion of 16α-OH-D to 16α-hydroxy-oestrone
The presumptive 16α-OH-D isolated from incubations 2, 3, 4 and 5 was reincubated at 37°C with 2 ml of a preparation of human placental microsomes (Ryan 1959) and 10 ml 0.067 M phosphate buffer (pH 7.0). Material from incubations 2, 3 and 4 was reincubated for 3 h in the presence of ATP, NAD and NADPH (10 µmoles of each). Materials from incubation 5 was reincubated for 1 h and only NADPH (1.2 µmole) was added. After these incubations, acetone (40 ml) was added to precipitate the proteins. Extraction procedures were the same as described above, as far as the extraction of the 50 % methanol fraction with ether. The residue from this ether fraction was chromatographed in system II.

RESULTS

Incubation of [14C]pregnenolone (cases A and B)
The »neutral steroid« fraction from incubation of case A was chromatographed in system I. Radioactivity was found associated with carrier 16α-OH-D and remained with this carrier after elution and rechromatography in system II. 16α-OH-D with associated radioactivity was eluted, acetylated and
rechromatographed sequentially in systems I and II. Radioactivity remained associated with carrier 16α-OH-D diacetate. In view of the thorough characterization of 16α-OH-D from case B already described (Shahwan et al. 1968) further purification was not carried out.

**Incubation of [3H]D (cases A and B)**

The »neutral steroid« fraction from incubations of both cases was chromatographed in system I and radioactivity was found with carrier 16α-OH-D and remained with this steroid on rechromatography in system II. 16α-OH-D and associated radioactivity were eluted and incubated with placental microsomes. This process effectively removed a contaminant since 16α-hydroxy-oestrone formed from carrier 16α-OH-D was found to be free from 3H after chromatography in system II. In consequence, conversion of [3H]D to [3H]16α-OH-D could not be demonstrated.

**Incubation of [3H]pregnenolone plus [14C]D (case C)**

i) **Identification of 16α-OH-D.** After chromatography of the »neutral steroid« fraction in system I, 3H and 14C were found associated with carrier 16α-OH-D. Radioactivity remained associated with this carrier after elution and rechromatography in system II. Incubation of the carrier 16α-OH-D and associated radioactivity with a placental microsome preparation and chromatography in system II yielded 16α-hydroxy-oestrone. This material was eluted, reduced with NaBH₄ and the product chromatographed in system II. 3H but not 14C was associated with the oestriol obtained. This material was eluted and dissolved in ether which was extracted with N NaOH. The alkaline phase was neutralized with N HCl and extracted with ether. The ether solution was washed with 8% NaHCO₃ solution and water. The residue after evaporation of the ether was chromatographed on Celite in system V. The distribution of carrier oestriol and 3H in the eluate is shown in Fig. 2. The 3H/14C ratios during the identification procedure of 16α-OH-D are shown in Table 2.

The yield of [3H]16α-OH-D calculated from the mean specific activities of the peak tubes was 1.5%/o.

ii) **Identification of D.** 3H and 14C from the »neutral steroid« fraction remained associated with carrier D throughout sequential chromatography in systems I, III and IV. This material was eluted, acetylated and rechromatographed in system III. Both isotopes remained associated with the acetylated derivative. The derivative was hydrolyzed and rechromatographed in system III. Carrier D, which still contained 3H and 14C, was eluted, incubated with a placental microsome preparation and an ether extract was chromatographed in system I. Carrier oestrione, derived from carrier D, contained both 3H and 14C. This material was chromatographed on a Celite column. Carrier oestrione, 3H and 14C were assayed in each fraction of eluate and were found to be
Fig. 2.
Distribution of $^3$H ($\Delta = \Delta$) and carrier steroid (••••) in the eluate from a partition column (system V) of oestriol derived from 16α-OH-D isolated after incubation of $[^3]$Hpregnenolone and $[^{14}]$C D.

Table 2.
$^3$H/$^{14}$C Ratios during identification procedure for 16α-OH-D isolated from incubation 5 (case C) with $[^3]$Hpregnenolone (19 µc, 24 µg) and $[^{14}]$C D (0.77 µc, 22 µg).

<table>
<thead>
<tr>
<th>Substrate mixture</th>
<th>3H Radioactivity DPM</th>
<th>14C Radioactivity DPM</th>
<th>$^3$H/$^{14}$C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate mixture</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16α-OH-D</td>
<td>I</td>
<td>41.8 X 10^6</td>
<td>1.7 X 10^6</td>
</tr>
<tr>
<td>16α-OH-D</td>
<td>II</td>
<td>297 300</td>
<td>3040</td>
</tr>
<tr>
<td>Oestriol</td>
<td>II</td>
<td>277 400</td>
<td>2720</td>
</tr>
<tr>
<td>Oestriol</td>
<td>V</td>
<td>91 600</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>81 710</td>
<td>0</td>
</tr>
</tbody>
</table>

present in a single peak (Table 3). The $^3$H/$^{14}$C ratios during the purification procedure are shown in Table 4. This data indicates that $[^3]$HD was formed from $[^3]$Hpregnenolone during the incubation. The yield of $[^3]$HD calculated from the specific activity of $[^3]$Hoestrone in the peak fractions was 1.7 \(\%\).
Table 3.
Specific activities (DPM/µg) of carrier oestrone eluted from partition column (system VI). Radioactive oestrone was derived from D isolated from incubation 5 (case C) with [3H]pregnenolone (19 µc, 24 µg) and [14C]D (0.77 µc, 22 µg).

<table>
<thead>
<tr>
<th>Fraction no.</th>
<th>Carrier oestrone (µg)</th>
<th>³H DPM</th>
<th>Specific activity ³H DPM/µg</th>
<th>¹⁴C DPM</th>
<th>Specific activity ¹⁴C DPM/µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.33</td>
<td>926</td>
<td>2806</td>
<td>462</td>
<td>1400</td>
</tr>
<tr>
<td>4</td>
<td>0.33</td>
<td>1289</td>
<td>3906</td>
<td>640</td>
<td>1939</td>
</tr>
<tr>
<td>5</td>
<td>2.22</td>
<td>15 875</td>
<td>7151</td>
<td>16 326</td>
<td>7354</td>
</tr>
<tr>
<td>6</td>
<td>1.22</td>
<td>8145</td>
<td>6676</td>
<td>8306</td>
<td>6808</td>
</tr>
<tr>
<td>7</td>
<td>0.22</td>
<td>1461</td>
<td>6641</td>
<td>1434</td>
<td>6518</td>
</tr>
<tr>
<td>8</td>
<td>0.16</td>
<td>589</td>
<td>3681</td>
<td>562</td>
<td>3513</td>
</tr>
<tr>
<td>9</td>
<td>0.11</td>
<td>158</td>
<td>1436</td>
<td>234</td>
<td>2127</td>
</tr>
<tr>
<td>10</td>
<td>0.11</td>
<td>158</td>
<td>1436</td>
<td>128</td>
<td>1164</td>
</tr>
</tbody>
</table>

Table 4.
³H/¹⁴C Ratios during identification procedure for D isolated from incubation 5 (case C) with [³H]pregnenolone (19 µc, 24 µg) and [¹⁴C]D 0.077 µc, 22 µg).

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Chromatography system</th>
<th>Radioactivity DPM</th>
<th>³H/¹⁴C</th>
</tr>
</thead>
<tbody>
<tr>
<td>D acetate</td>
<td>III</td>
<td>7.55 X 10⁴</td>
<td>7.87 X 10⁴</td>
</tr>
<tr>
<td>D</td>
<td>III</td>
<td>5.42 X 10⁴</td>
<td>5.72 X 10⁴</td>
</tr>
<tr>
<td>Oestrone</td>
<td>I</td>
<td>3.74 X 10⁴</td>
<td>3.85 X 10⁴</td>
</tr>
<tr>
<td>Oestrone</td>
<td>VI</td>
<td>2.55 X 10⁴</td>
<td>2.61 X 10⁴</td>
</tr>
</tbody>
</table>

Control incubations
Examination of the »neutral steroid« fractions derived from the control incubations of [¹⁴C]pregnenolone and [³H]D showed that radioactivity was only associated with carrier pregnenolone and D respectively. No radioactivity was associated with carrier 16α-OH-D.

DISCUSSION

Identification of radioactive metabolites by reverse isotope dilution techniques relies on the failure to separate a particular metabolite from authentic material. Consequently, thorough purification of any metabolite must be carried out and
the results examined critically. The procedure for the purification of 16α-OH-D from the incubation with [3H]pregnenolone and [14C]D comprised sequential chromatography in two solvent systems, derivative formation with placental enzymes, reduction of the derivative and, finally, partition column chromatography. During this procedure, 14C, derived from [14C]D and present in the early fractions, was separated from authentic 16α-OH-D. In contrast, 3H, derived from [3H]pregnenolone, could not be separated from carrier 16α-OH-D. Therefore, 16α-OH-D was derived from [3H]pregnenolone but not from [14C]D. Additional support for this finding was the failure to isolate [3H]16α-OH-D after incubation of [3H]D with adrenal tissue from other anencephalic infants. Our results, therefore, provide strong support for the existence of a pathway of biosynthesis for 16α-OH-D via 16α-hydroxy C21 steroids as envisaged by Kirschner et al. (1966).

A search was made for C21 intermediates between pregnenolone and 16α-OH-D – for example, 16α-hydroxy-pregnenolone and 3β,16α,17α-trihydroxy-pregn-5-en-20-one. The latter compound was not detected. A small quantity of 3H was found associated with carrier 16α-hydroxy-pregnenolone and remained with this steroid through sequential chromatography and acetylation. Lack of material prevented further characterization. Villee et al. (1962) have reported briefly that 16α-hydroxy-pregnenolone was an important metabolite of pregnenolone incubated with adrenal tissue from an anencephalic infant. Hydroxylation of pregnenolone at C-16α by the foetal adrenal, and especially by the definitive zone, therefore appears possible, in vitro. Some substrate specificity for the 16α-hydroxylase of this tissue may operate, since pregnenolone, but not D, served as substrate. The detection of [3H]D and the absence of [14C]16α-OH-D from the incubation of [3H]pregnenolone and [14C]D emphasizes this feature.

Extrapolation of our results, obtained in vitro, to the situation in vivo may not be justified. The incubations reported were carried out with «free» steroids, whereas steroid sulphate esters may play a more important role, especially in the foetus, which has little sulphatase activity (Eberlein 1965).

The quantities of endogenous pregnenolone and D in the tissue were not measured. Therefore, no indication of the mass of substrate converted to 16α-OH-D is available. It is unlikely that a pathway in the definitive zone of the foetal adrenal, such as that described in vitro, plays a significant role in the biosynthesis of 16α-OH-D and its sulphate and, ultimately, of oestriol, in normal pregnancy. The measurements reported by Easterling et al. (1966) and by Frandsen & Stakemann (1961) demonstrate that the major source of these steroids, or their precursors, in normal pregnancy is the foetal zone. Nevertheless, the pathway from pregnenolone to 16α-OH-D may contribute to the measurable quantities of 16α-OH-D and 16α-OH-DS found in the cord blood of newborn anencephalic infants. Since 16α-OH-D is efficiently converted to
oestriol by the placenta (Dell’Acqua et al. 1967), this pathway could provide precursors for oestriol excreted in pregnancy with an anencephalic foetus. Oestriol excretion, although substantially reduced in this condition, is some 100 times greater than in non-pregnancy. Further experiments are required to decide whether a similar pathway exists in the maternal system.

The metabolism of pregnenolone to 16α-OH-D detected in predominantly definitive zone tissue may also contribute to the production of 16α-OH-D which has been identified in urine of newborn infants by Reynolds (1965) and by Shackleton & Mitchell (1967).

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