CONVERSION OF ANDROGENS INTO OESTROGENS
BY HYDATIDIFORM MOLES IN VITRO

By
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and Maria Siemerink

ABSTRACT

[1,2-3H] and [7α-3H] testosterone* and [4-14C] androstenedione incubated with hydatidiform mole tissue are converted into oestrone and oestradiol. No conversion into oestriol was observed. The radiometabolites were purified using TLC in different systems and crystallization to a constant specific activity and/or 14C/3H ratio. Appropriate corrections were made for endogenous production of steroids during the incubation.

From time-experiments with [4-14C] androstenedione and [7α-3H] or [1,2-3H] testosterone it appears, that androstenedione is a more effective precursor than testosterone for oestrone and oestradiol. Equilibrium oestrone ↔ oestradiol is established more rapidly than androstenedione ↔ testosterone. Conversion of androstenedione into oestrone is more effective than conversion of testosterone into andro-

* The following trivial names of steroids and abbreviations are used in this paper: dehydroepiandrosterone (DHA), 3-hydroxy-androst-5-en-17-one; testosterone, 17β-hydroxy-androst-4-en-3-one; androstenedione, androst-4-ene-3,17-dione; oestrone, 3-hydroxy-oestra-1,3,5(10)-triene-17-one; 17β-oestradiol, oestra-1,3,5(10)-triene-3,17β-diol; oestriol, oestra-1,3,5(10)-triene-3,16α,17β-triol; [1,2-3H] testosterone, [1,2-3H] 17β-hydroxy-androst-4-en-3-one; [7α-3H] testosterone, [7α-3H] 17β-hydroxy-androst-4-en-3-one; [4-14C] androstenedione, [4-14C] androst-4-ene-3,17-dione.
stenedione. Data are reported in agreement with the hypothesis of a direct pathway from testosterone to oestradiol in the shorter periods of incubation.

Determination of the endogenous pools of oestrone, oestradiol, testosterone and androstenedione synthesized during the incubations indicates that oestrone is the most important steroid produced in vitro.

It has been shown that a 16-hydroxylated neutral metabolite (Bolté et al. 1964a,b,c), possibly formed from dehydroepiandrosterone sulphate circulating in the foetus (Ryan 1959), and/or a 16-hydroxylated phenolic intermediate formed by the foetus from placental oestrone (Ryan 1959) can be converted into oestriol by the placenta. Interruption of the foeto-placental connection results in a sudden drop in the excretion of oestrogens by the mother, whereas artificial perfusion of the placenta in situ with maternal blood maintained the excretion at preoperative levels (Cassmer 1959). The decrease in the excretion of oestriol was particularly pronounced in these experiments.

The experimental situation of Cassmer (1959) exists as a natural experiment: in hydatidiform mole pregnancy there is no circulation in the chorionic villi and the foetus is absent. Thus steroidogenesis in molar pregnancy is of special interest.

A considerable excretion of oestrone, oestradiol and oestriol, higher than in non-pregnant women but lower than in women with a normal pregnancy has been demonstrated in patients with vesicular moles (Frandsen & Stakemann 1964; Kock et al. 1965; Houtzager 1968).

Oestrone and oestradiol but not oestriol are produced in high yields by vesicles of hydatidiform moles incubated in vitro in the presence of dehydroepiandrosterone (Van Leusden & Villee 1966a,b; Van Leusden 1966, 1967). Oestriol, appearing in the urine of patients with hydatidiform moles as in the urine of non-pregnant women, is derived primarily from the metabolism of oestrone and oestradiol in the maternal compartment (Van Leusden & Villee 1966a; Houtzager 1968; Houtzager & Van Leusden 1969).

As metabolic conversion of dehydroepiandrosterone into oestrone and oestradiol in hydatidiform moles has been established, the question arose whether androgens other than dehydroepiandrosterone would be aromatized in molar tissue.

In the present investigation tissue samples of hydatidiform moles were incubated in vitro with [7α-3H] or [1,2-3H] testosterone and/or [4-14C] androstenedione in order to study possible metabolic conversion of these steroids into oestrone, oestradiol and oestriol. The question whether testosterone or androstenedione would be a more effective precursor is investigated in time incubations with [7α-3H] or [1,2-3H] testosterone and [4-14C] androstenedione followed by isolation and purification of oestrone and oestradiol. In the same
experiments metabolic conversion of androstenedione into testosterone and vice versa was measured as compared with oestrone $\rightarrow$ oestradiol.

As during the incubations there might be an influence of endogenous production of steroids, experiments were designed to measure their possible contribution to this endogenous production of steroids. From the data thus obtained appropriate corrections for percentages of conversions were applied. The question, whether oestradiol or oestrone is the most important steroid produced \textit{in vitro} could thus be answered.

\textbf{MATERIALS AND METHODS}

\[1,2^3\text{H}\] \text{testosterone}, \[7\alpha^3\text{H}\] \text{testosterone and} \[4^{-14}\text{C}\] \text{androstenedione} were purchased from New-England Nuclear Corporation. The substrates were subjected to thin layer chromatography on silica gel \(G\), using benzene:ethanol (9:1) as the liquid phase. No significant amounts of impurities were detected. Following chromatography, evidence of radiochemical homogeneity was obtained by crystallization of a portion of the radioactive material with authentic carrier steroids. The specific activities of the mother liquor and of the crystals were identical and were the same as that calculated for the mixture.

The radioactivity of the samples was measured in a Nuclear-Chicago scintillation spectrometer (M I), with external standardization. Samples containing both tritium and Carbon-14 were assayed by the procedure of Okita et al. (1957).

Samples of molar tissue 1000 \((I)\), 1200 \((II)\), 1250 \((III)\) and 1200 g \((IV)\) respectively, from pregnancies of 9, 11, 12 and 12 weeks were obtained immediately after delivery. From each 10 g portions of tissue were dissected and placed in 10 ml of incubation medium in a 125 ml glass-stoppered Erlenmeyer flask (experiments A, B, D, E).

The incubation medium consisted of Krebs-Ringer-phosphate buffer, pH 7.4, containing 11.1 mM glucose, 5 \(\mu\)M DPN, 300 \(\mu\)M nicotinamide, 10 \(\mu\)M TPN and 10 \(\mu\)M ATP. The flasks were incubated in an atmosphere of air during 5–240 min (see Table 1) at 37°C with shaking.

In experiments C and F 40 g molar tissue was homogenized for 1 min in 29.32 ml buffer in a motor-driven homogenizer (ATO-Mix). 17.37 g homogenate (corresponding to 10 g wet molar tissue) was used in each incubation experiment Ca, b, c; Fa, b (Table 1). After addition of cofactors the total volume was 20 ml.

\([4^{-14}\text{C}\] \text{androstenedione and/or} \[1,2^3\text{H}\] \text{testosterone} (Table 1) in ethanol had been placed in the flask and the solvent had been dried under a stream of nitrogen before the incubation medium and tissues were added. In experiments C and F \([1,2^3\text{H}\] \text{testosterone was used} and not \([7\alpha^3\text{H}\] \text{testosterone} (Table 1).

After incubation, the incubation medium and the molar tissue were placed in a motor-driven homogenizer (ATO-Mix) and extracted with ethanol-ether-acetone (1:4:4, v/v). Before the initial extraction known amounts (Table 1) of recrystallized oestrone, oestradiol, oestriol, androstenedione and testosterone were added to the combined tissue and medium to measure the recovery of the respective steroids. The incubation medium and tissue were placed in the homogenizer, the carrier steroids were added and the mixture was homogenized for 3 min in 30 ml of ethanol-ether-acetone (1:4:4, v/v).

The contents were transferred and the homogenizer was rinsed with 5 successive 10 ml portions of ethanol-ether-acetone. The homogenate and rinses were combined.
Table 1.
Survey of the incubation experiments.

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Radioactive precursors added</th>
<th>Standard steroids added after incubation before extraction (µg)</th>
<th>Incubation time min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>OE₁</td>
<td>OE₂</td>
</tr>
<tr>
<td>A</td>
<td>[1,2-³H] testosterone</td>
<td>920</td>
<td>930</td>
</tr>
<tr>
<td>B</td>
<td>[4-¹⁴C] androstenedione</td>
<td>1040</td>
<td>1065</td>
</tr>
<tr>
<td></td>
<td>[7α-³H] testosterone</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ca</td>
<td>[4-¹⁴C] androstenedione</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>[7α-³H] testosterone</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cb</td>
<td>[4-¹⁴C] androstenedione</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>[7α-³H] testosterone</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cc</td>
<td>[4-¹⁴C] androstenedione</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>[7α-³H] testosterone</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Da</td>
<td>[4-¹⁴C] androstenedione</td>
<td>920</td>
<td>930</td>
</tr>
<tr>
<td></td>
<td>[1,2-³H] testosterone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Db</td>
<td>[4-¹⁴C] androstenedione</td>
<td>920</td>
<td>930</td>
</tr>
<tr>
<td></td>
<td>[1,2-³H] testosterone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dc</td>
<td>[4-¹⁴C] androstenedione</td>
<td>920</td>
<td>930</td>
</tr>
<tr>
<td></td>
<td>[1,2-³H] testosterone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dd</td>
<td>[4-¹⁴C] androstenedione</td>
<td>920</td>
<td>930</td>
</tr>
<tr>
<td></td>
<td>[1,2-³H] testosterone</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Compound</td>
<td>Ea</td>
<td>Eb</td>
</tr>
<tr>
<td>---</td>
<td>---------------------------------</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>[4-14C] androstenedione</td>
<td>1050</td>
<td>1140</td>
</tr>
<tr>
<td></td>
<td>[1,2-3H] testosterone</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>[4-14C] androstenedione</td>
<td>1050</td>
<td>1140</td>
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<tr>
<td></td>
<td>[1,2-3H] testosterone</td>
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<td></td>
<td>[4-14C] androstenedione</td>
<td>1050</td>
<td>1140</td>
</tr>
<tr>
<td></td>
<td>[1,2-3H] testosterone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fb</td>
<td>[7α-3H] testosterone</td>
<td>510</td>
<td>1506</td>
</tr>
<tr>
<td>Fa</td>
<td>[7α-3H] testosterone</td>
<td>1580</td>
<td>502</td>
</tr>
</tbody>
</table>

Expts A, D and E with mole I
Expt. B with mole II
Expt. C with mole III
Expt. F with mole IV
and filtered through Whatman No. 1 paper. The filtrate was taken to dryness under nitrogen at 30°C. The pH was adjusted to pH 8.3. The filtrate was partitioned between n-hexane and 90 % methanol. This was carried out in a counter current manner using 5 transfers.

The methanol phase was taken to dryness and partitioned between water and ether. The ether fraction was evaporated under nitrogen and partitioned between toluene and N-NaOH. The pH of the NaOH fraction was adjusted to 10.3 and subsequently this fraction was extracted with ether. This ether fraction was taken to dryness under nitrogen and subjected to thin layer chromatography on silica gel G using benzene:ethanol (9:1) as the liquid phase. Subsequently the regions of oestrone, oestradiol and oestriol were chromatographed on silica gel G using chloroform:acetone (8:2) as the liquid phase. The regions of the oestrogens were eluted and the amount of each recovered was determined according to Struck (1961). Known amounts of standard oestrone, oestradiol and oestriol were added for recrystallizations from different solvents.

In experiments C and F the toluene fraction was purified by thin layer chromatography in chloroform:ethyl acetate (8:2), benzene:ethanol (9:1), methylene chloride:acetone (8:2) and chloroform:acetone (8:2).

After elution of the androstenedione and testosterone regions, the amounts recovered, were determined by absorption at 240 nm in ethanol in a Beckman DU2 spectrophotometer. Subsequently known amounts of standard androstenedione and testosterone were added for recrystallization from different solvents.

For recrystallization the least possible amount of warm solvent to keep the radio-metabolite in solution was used. One drop of water was added and the mixture was kept at 0-4°C in order to obtain slow crystallization, care being taken that a real crystallization and not precipitation took place.

The silica gel G used for TLC was washed 5 times with ethanol before use until no UV detectable material was present in the eluate.

Standard steroids were purchased from Sigma Chemical Comp. and recrystallized before use.

RESULTS

Oestriol

In experiments A, B, D and E no formation of 3H and/or 14C labelled oestriol could be demonstrated.

The oestriol region after TLC in benzene:ethanol (9:1) and chloroform:acetone (8:2) in experiments A, B, D a, b, c, d and E a, b, c, contained 300-600 µg of oestriol.

Standard oestriol was added for recrystallizations. During crystallization all radioactivity disappeared from the crystals into the supernatant.

Conversion of testosterone into oestrone and oestradiol (experiment A)

After TLC of the ether fraction in benzene:ethanol (9:1) and chloroform:acetone (8:2), the oestrone and oestradiol regions were eluted from the plates

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Table 2.
Experiment A.
Specific activity of oestrone and 17β-oestradiol isolated from incubation of hydatidiform mole (I) with [1,2-3H] testosterone (7384 × 10³ dpm; specific activity: 0.25 mCi/1.5 µg).

<table>
<thead>
<tr>
<th>Crystals</th>
<th>Solvent</th>
<th>Oestrone</th>
<th>Oestradiol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>³H, dpm/mg</td>
<td>³H, dpm/mg</td>
</tr>
<tr>
<td>first</td>
<td>methanol</td>
<td>31 800</td>
<td>68 200</td>
</tr>
<tr>
<td>second</td>
<td>acetone</td>
<td>29 300</td>
<td>67 300</td>
</tr>
<tr>
<td>third</td>
<td>ethanol</td>
<td>28 900</td>
<td>65 000</td>
</tr>
<tr>
<td>mother liquor</td>
<td></td>
<td>28 400</td>
<td>63 800</td>
</tr>
</tbody>
</table>

920 µ carrier oestrone added before initial extraction;
6300 µg standard oestrone added to oestrone fraction from TLC (615 µg).
930 µ carrier oestradiol added before initial extraction;
7300 µg standard oestradiol added to oestradiol fraction from TLC (611 µg).

and after addition of standard oestrone or standard oestradiol, the radiometabolites were crystallized to a constant specific activity (Table 2).

Oestrone

The percentage of conversion from [1,2-3H] testosterone into oestrone in experiment A (Table 2) can be calculated from the data presented in Table 2. It would be assumed that during aromatisation [1,2-³H] testosterone would lose ³H; in that case the real conversion into oestrone and oestradiol should be corrected; therefore the factor 2 is introduced*.

Thus % conversion:
\[
\frac{615 + 6300}{615} \times 920 \times 29.1 \times 100 \times 2 = 8.2
\]

Oestradiol

Calculated in a similar manner the percentage of conversion from [1,2-³H]-testosterone into oestradiol is 21.2.

* It is realized that whether this factor actually is 2 can depend on the positions of ³H, which may be α or β; as long as this has not been proved, it is assumed that the factor is 2.
Conversion of androstenedione into oestrone and oestradiol (experiment B)

**Oestrone**

After TLC of the ether fraction in benzene:ethanol (9:1) the oestrone region was rechromatographed in chloroform:acetone (8:2).

After addition of a known amount of standard oestrone, the radiometabolite was crystallized to a constant specific activity (Table 3).

The percentage of conversion from [4-14C] androstenedione into oestrone in experiment B (Table 3) can be calculated from the data presented in Table 3 and is 3.1.

**Oestradiol**

After TLC of the ether fraction in benzene:ethanol (9:1), the oestradiol region was eluted and rechromatographed in chloroform:acetone (8:2).

After addition of a known amount of standard oestradiol the radio-metabolite was crystallized to a constant specific activity (Table 3). The percentage of conversion from [4-14C] androstenedione into oestradiol in experiment B (Table 3) is 3.8.

Time incubations with [4-14C] androstenedione and [7α-3H] testosterone as precursors (experiment C)

Equimolar amounts of the substrates were present at the start of the experiment (Tables 4 and 5).

**Table 3.**

Experiment B.

Specific activity of oestrone and 17β-oestradiol isolated from incubation of hydatidiform mole (II) with [4-14C] androstenedione (8278 × 10^3 DPM; specific activity: 10 μCi/49.3 μg).

<table>
<thead>
<tr>
<th>Crystals</th>
<th>Solvent</th>
<th>Oestrone</th>
<th>Oestradiol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14C, DPM/mg</td>
<td></td>
<td>14C, DPM/mg</td>
</tr>
<tr>
<td>first</td>
<td>methanol</td>
<td>6680</td>
<td>7170</td>
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<tr>
<td>second</td>
<td>acetone</td>
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<td>7600</td>
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<td>third</td>
<td>ethanol</td>
<td>6490</td>
<td>7680</td>
</tr>
<tr>
<td>mother liquor</td>
<td></td>
<td>6210</td>
<td>7200</td>
</tr>
</tbody>
</table>

1040 μg carrier oestrone added before initial extraction;
16 672 μg standard oestrone added to oestrone fraction from TLC (445 μg).
1065 μg carrier oestradiol added before initial extraction;
15 745 μg standard oestradiol added to oestradiol fraction from TLC (416 μg).
Table 4.

Experiment C.

Specific activity of oestrone isolated from incubation of hydatidiform mole (III) with [4-\(^{14}\)C] androstenedione (22.530 \times 10^6 \text{ DPM} \text{ in } 500 \, \mu\text{g}) and [7α-\(^3\)H] testosterone (42.770 \times 10^6 \text{ DPM} \text{ in } 504 \, \mu\text{g}).

<table>
<thead>
<tr>
<th>Crystals</th>
<th>Solvent</th>
<th>Ca (120 min)</th>
<th></th>
<th>Cb (180 min)</th>
<th></th>
<th>Cc (240 min)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(^{14})C, \text{ DPM/mg}</td>
<td>(^3)H, \text{ DPM/mg}</td>
<td>(^{14})C, \text{ DPM/mg}</td>
<td>(^3)H, \text{ DPM/mg}</td>
<td>(^{14})C, \text{ DPM/mg}</td>
<td>(^3)H, \text{ DPM/mg}</td>
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<td>9350</td>
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<td>2470</td>
<td>1580</td>
<td>11 800</td>
<td>7290</td>
<td>9500</td>
<td>5900</td>
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</table>
Table 5.
Experiment C.
Specific activity of oestriol isolated from incubation of hydatidiform mole (III) with [4-\textsuperscript{14}C] androstenedione (22 530×10\textsuperscript{3} DPM in 500 \textmu g) and [7α-\textsuperscript{3}H] testosterone (42 770×10\textsuperscript{3} DPM in 504 \textmu g).

<table>
<thead>
<tr>
<th>Crystals</th>
<th>Solvent</th>
<th>Ca (120 min)</th>
<th></th>
<th>Cb (180 min)</th>
<th></th>
<th>Cc (240 min)</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>\textsuperscript{14}C, DPM/\textmu g</td>
<td>\textsuperscript{3}H, DPM/\textmu g</td>
<td>\textsuperscript{14}C, DPM/\textmu g</td>
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<td>\textsuperscript{14}C, DPM/\textmu g</td>
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</tbody>
</table>

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Before initial extraction in experiment C no standard oestrone and oestradiol had been added (Table 1).

\[^{14}C \text{ and } ^{3}H \text{ in oestrone and oestradiol}\]

After separation of oestrone and oestradiol by TLC of the ether fraction in benzene:ethanol (9:1) and chloroform:acetone (8:2), addition of standard oestrone of standard oestradiol, the radiometabolites were crystallized to a constant specific activity (Tables 4 and 5).

The \[^{14}C/^{3}H\] ratios of oestrone and oestradiol were determined after incubation during 120, 180 and 240 min (Table 6).

\[\text{Determination of } ^{14}C/^{3}H \text{ in testosterone and androstenedione}\]

After TLC of the toluene fractions in chloroform:ethyl acetate (8:2), benzene:ethanol (9:1), methylene chloride:acetone (8:2) and chloroform:acetone (8:2) the \[^{3}H-^{14}C^{*}\] testosterone and \[^{3}H-^{14}C^{*}\] androstenedione regions were eluted from the plates.

After addition of standard testosterone and standard androstenedione respectively, the radiometabolites were crystallized to a constant specific activity.

The \[^{14}C/^{3}H\] ratios of \[^{3}H-^{14}C\] testosterone and \[^{3}H-^{14}C\] androstenedione were determined after incubation during 120, 180 and 240 min (Table 7).

Time incubations with \[4-^{14}C\] androstenedione and \[1,2-^{3}H\] testosterone for intervals shorter than 120 min (experiments D and E)

It should be noted that in experiments D and E, the substrates were not present at the start of the experiments in equimolar amounts (see Tables 8 and 9).

\[\text{Table 6.}\]

Expt. C. \[^{14}C/^{3}H\] in oestrone and oestradiol isolated after incubation of homogenized molar-tissue (mole III) with \[4-^{14}C\] androstenedione (22,530 \times 10^3 \text{ dpm in 500 } \mu\text{g}) and \[7a-^{3}H\] testosterone (42,770 \times 10^3 \text{ dpm in 504 } \mu\text{g}) during 120, 180 and 240 min.

Starting ratio 0.5.

<table>
<thead>
<tr>
<th></th>
<th>expt. Ca 120 min</th>
<th>expt. Cb 180 min</th>
<th>expt. Cc 240 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{14}C/^{3}H) in oestrone</td>
<td>1.6</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>(^{14}C/^{3}H) in oestradiol</td>
<td>1.6</td>
<td>1.6</td>
<td>1.5</td>
</tr>
</tbody>
</table>

* Strictly speaking the location of the radioactivity in the products has not been demonstrated and thus, for example, it is more correct to use \[^{3}H-^{14}C\] testosterone and not \[^{7}a-^{3}H-4-^{14}C\] testosterone.

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Table 7.
Expt. C. $^{14}$C/$^{3}$H of purified $[^{3}$H-$^{14}$C] testosterone and $[^{3}$H-$^{14}$C] androstenedione, isolated after incubation of homogenized molar-tissue (mole III) with equimolar amounts $[^{4}$-$^{14}$C] androstenedione (22 530 $\times$ 10$^{3}$ dpm in 500 $\mu$g) and $[^{7a}$-$^{3}$H] testosterone (42 770 $\times$ 10$^{9}$ dpm in 504 $\mu$g) during 120, 180 and 240 min.

<table>
<thead>
<tr>
<th></th>
<th>Expt. Ca 120 min</th>
<th>Expt. Cb 180 min</th>
<th>Expt. Cc 240 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{14}$C/$^{3}$H in $[^{3}$H-$^{14}$C] testosterone</td>
<td>0.3</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>$^{14}$C/$^{3}$H in $[^{3}$H-$^{14}$C] androstenedione</td>
<td>0.9</td>
<td>0.7</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Table 8.
Expts. D and E. Percentage of conversion from $[^{4}$-$^{14}$C] androstenedione (8015 $\times$ 10$^{3}$ dpm; 10 $\mu$Ci/63.4 $\mu$g) and $[^{1,2}$-$^{3}$H] testosterone (13 525 $\times$ 10$^{3}$ dpm; 250 $\mu$Ci/1.5 $\mu$g) into oestrone and oestradiol in incubation experiments during 5, 10, 20, 30, 60, 120 and 240 min. Starting ratio: 0.6.

<table>
<thead>
<tr>
<th>Incubation time in min</th>
<th>Conversion % into oestrone</th>
<th>Conversion % into oestrone</th>
<th>Conversion % into oestrone</th>
<th>Conversion % into oestrone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A-$^{14}$C</td>
<td>T-$^{3}$H</td>
<td>A-$^{14}$C</td>
<td>T-$^{3}$H</td>
</tr>
<tr>
<td>Expt. Da 5</td>
<td>0.13 (0.13)*</td>
<td>0.02 (0.02)</td>
<td>0.19 (0.19)</td>
<td>0.04 (0.04)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expt. Db 10</td>
<td>0.31 (0.32)</td>
<td>0.04 (0.04)</td>
<td>0.46 (0.46)</td>
<td>0.06 (0.06)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expt. Dc 20</td>
<td>1.01 (1.07)</td>
<td>0.14 (0.14)</td>
<td>0.48 (0.48)</td>
<td>0.08 (0.08)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expt. Dd 30</td>
<td>1.21 (1.32)</td>
<td>0.26 (0.28)</td>
<td>2.52 (2.54)</td>
<td>0.44 (0.44)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expt. Ea 60</td>
<td>0.92 (1.06)</td>
<td>0.32 (0.38)</td>
<td>8.00 (8.07)</td>
<td>2.30 (2.30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expt. Eb 120</td>
<td>2.17 (2.84)</td>
<td>1.06 (1.40)</td>
<td>27.84 (28.32)</td>
<td>14.14 (14.34)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expt. Ec 240</td>
<td>4.72 (7.65)</td>
<td>2.10 (3.40)</td>
<td>5.61 (5.77)</td>
<td>2.32 (2.38)</td>
</tr>
</tbody>
</table>

* The data in brackets are the conversion percentages corrected for the endogenous production of steroids during the incubation (see expt. F).

Conversion into oestrone and oestradiol

After separation of oestrone and oestradiol by TLC of the ether fraction in benzene:ethanol (9:1) and chloroform:acetone (8:2), addition of standard oestrone and standard oestradiol respectively, the radiometabolites in this incubation series were crystallized to a constant specific activity.

The percentages of conversion from $[^{1,2}$-$^{3}$H] testosterone and $[^{4}$-$^{14}$C]-androstenedione to oestrone and oestradiol in experiment D and E were calculated and are presented in Table 8.
Table 9.
Expts. D and E. $^{14}$C/$^3$H of oestrone and oestradiol, isolated after incubation of molar tissue (mole I) with [4-$^{14}$C] androstenedione ($8015 \times 10^3$ dpm; 10 µCi/63.4 µg) and [1,2-$^3$H] testosterone ($13525 \times 10^3$ dpm; 250 µCi/1.5 µg) during 5, 10, 20, 30, 60, 120 and 240 min. Starting ratio: 0.6.

<table>
<thead>
<tr>
<th>Incubation time in minutes</th>
<th>$^{14}$C/$^3$H of oestrone</th>
<th>$^{14}$C/$^3$H of oestradiol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment Da</td>
<td>5</td>
<td>3.3</td>
</tr>
<tr>
<td>Experiment Db</td>
<td>10</td>
<td>4.1</td>
</tr>
<tr>
<td>Experiment Dc</td>
<td>20</td>
<td>4.1</td>
</tr>
<tr>
<td>Experiment Dd</td>
<td>30</td>
<td>3.3</td>
</tr>
<tr>
<td>Experiment Ea</td>
<td>60</td>
<td>1.8</td>
</tr>
<tr>
<td>Experiment Eb</td>
<td>120</td>
<td>1.1</td>
</tr>
<tr>
<td>Experiment Ec</td>
<td>240</td>
<td>1.2</td>
</tr>
</tbody>
</table>

$^{14}$C and $^3$H in oestrone and oestradiol

The $^{14}$C/$^3$H of oestrone and oestradiol were determined after incubation during 5, 10, 20, 30, 60, 120 and 240 min; appropriate corrections were applied for losses of $^3$H during aromatization of [1,2-$^3$H] testosterone (Table 9).

$^{14}$C and $^3$H in testosterone and androstenedione

After TLC of the toluene fraction in chloroform:ethyl acetate (8:2), benzene:ethanol (9:1), methylene chloride:acetone (8:2) and chloroform:acetone (8:2), the [$^3$H-$^{14}$C] testosterone and [$^3$H-$^{14}$C] androstenedione regions were eluted from the plates. After addition of standard testosterone and standard androstenedione respectively, the radiometabolites were crystallized to a constant specific activity. No conversion percentages were calculated (no standard testosterone and androstenedione had been added after incubation before the extraction).

The $^{14}$C/$^3$H ratios of [$^3$H-$^{14}$C] testosterone and [$^3$H-$^{14}$C] androstenedione were determined after incubation during 5, 10, 20, 30, 60, 120 and 240 min (Table 10).

Determination of endogenous production of androstenedione, testosterone, oestrone and oestradiol in molar tissue during in vitro incubations (experiment F)

In extracts of 10 g of hydatidiform mole tissue after purification as described no endogenous amounts of androstenedione, testosterone, oestrone and oestradiol could be demonstrated.
Table 10.
Expts. D and E. $^{14}C/^{3}H$ of purified [2H-14C] testosterone and [3H-14C] androstenedione, isolated after incubation of molar tissue (mole I) with [4-14C] androstenedione (8015 $\times 10^{3}$ DPM; 10 $\mu$Ci/63.4 $\mu$g) and [1,2-3H] testosterone (13,525 $\times 10^{3}$ DPM; 250 $\mu$Ci/1.5 $\mu$g) during 5, 10, 20, 30, 60, 120 and 240 min. Starting ratio: 0.6.

<table>
<thead>
<tr>
<th>Incubation time in minutes</th>
<th>$^{14}C/^{3}H$ of [2H-14C] testosterone</th>
<th>$^{14}C/^{3}H$ of [3H-14C] androstenedione</th>
</tr>
</thead>
<tbody>
<tr>
<td>Da</td>
<td>5</td>
<td>0.8</td>
</tr>
<tr>
<td>Db</td>
<td>10</td>
<td>0.05</td>
</tr>
<tr>
<td>Dc</td>
<td>20</td>
<td>0.05</td>
</tr>
<tr>
<td>Dd</td>
<td>30</td>
<td>0.05</td>
</tr>
<tr>
<td>Ea</td>
<td>60</td>
<td>0.05</td>
</tr>
<tr>
<td>Eb</td>
<td>120</td>
<td>0.2</td>
</tr>
<tr>
<td>Ec</td>
<td>240</td>
<td>0.2</td>
</tr>
</tbody>
</table>

When tissues of hydatidiform moles are incubated in Krebs-Ringer phosphate buffer, they continue to synthesize steroids regardless of whether exogenous precursors have been added to the medium.

If any steroid in an incubation with a radioactive steroid precursor should be produced endogenously in amounts that are large enough to alter significantly conversion values obtained by radio-isotope dilution analysis, then the principles of this analysis do not hold and correction must be made, according to the method described by Cameron & Griffiths (1968).

In order to perform this correction, the experimental results of the incubation experiment Fa of the present series were combined in simultaneous equations with those from experiment Fb, in which the same amount of molar tissue homogenate was incubated.

Larger amounts of carrier steroids (oestrone, oestradiol, testosterone and androstenedione) were added to experiment Fb as compared with Fa thus enabling the following equations to be solved (see page 31).

The values for the endogenous production of oestrone, oestradiol, testosterone and androstenedione calculated for the 240 min incubation were extrapolated back to give approximate values for endogenous production during the shorter time-intervals.

40 g molar tissue was homogenized in 29–32 ml buffer in a motor-driver homogenizer (ATO-Mix).

For each experiment Fa and Fb, 17.37 g of this homogenate was taken, corresponding to 10 g wet molar tissue.

In experiment Fa the homogenized tissue was incubated (240 min) with [7a-3H] testosterone (14,290 $\times 10^{3}$ DPM; 250 $\mu$Ci/5 $\mu$g).
Equations used for corrections (see page 30).

\[
\text{true } \% \text{ conversion } = \frac{\mu g \text{ standard steroid } Fa + \mu g \text{ steroid fraction } Fa \text{ from TLC}}{\mu g \text{ steroid fraction } Fa \text{ from TLC}} \times \frac{\mu g \text{ carrier steroid } Fa + \mu g \text{ endog.}}{\times \text{ S. A. Fa}} \times 100
\]

\[
\text{true } \% \text{ conversion } = \frac{\mu g \text{ standard steroid } Fb + \mu g \text{ steroid fraction } Fb \text{ from TLC}}{\mu g \text{ steroid fraction } Fb \text{ from TLC}} \times \frac{\mu g \text{ carrier steroid } Fb + \mu g \text{ endog.}}{\times \text{ S. A. Fb}} \times 100
\]

S A. Fa = specific activity of a steroid isolated from Fa incubation, S. A. Fb = specific activity of a steroid isolated from Fb incubation, \( \mu g \) standard steroid Fa = \( \mu g \) of the steroid added to the steroid fraction from TLC in Fa, \( \mu g \) standard steroid Fb = the steroid added to the steroid fraction from TLC in Fb, \( \mu g \) carrier steroid Fa = the carrier steroid added before initial extraction, \( \mu g \) carrier steroid Fb = the carrier steroid added before initial extraction.
In experiment Fa 510 µg oestrone, 1506 µg oestradiol, 494 µg testosterone and 2084 µg androstenedione were added after the incubations but before extractions.

In experiment Fb, the homogenized molar tissue was incubated with [7α-3H]-testosterone (14 290 × 10^3 DPM; 250 µCi/5 µg) for a similar period. After incubation the following amounts of carrier steroids were added: 1530 µg oestrone, 502 µg oestradiol, 1482 µg testosterone and 500 µg androstenedione.

**Determination of endogenous production of oestrone**

After TLC of the ether fraction in benzene/ethanol (9:1) and chloroform:acetone (8:2) standard oestrone was added and the radiometabolite was crystallized to a constant specific activity (Table 11).

The endogenous production of oestrone (XOE_f) in experiment F can be calculated from the data in Table 11 as follows:

\[
\begin{align*}
\text{Fa} & \quad \frac{5200 + 238}{238} \times (0.51 + \text{XOE}_f) \times 444 000 \\
\text{Fb} & \quad \frac{5200 + 626}{626} \times (1.53 + \text{XOE}_f) \times 580 000 \\
\end{align*}
\]

\[
14 290 \times 10^3 \\
14 290 \times 10^3
\]

Knowing the endogenous production of oestrone (XOE_f = 650 µg) the corrected percentage of conversion from [7α-3H] testosterone into oestrone can be calculated.

**Table 11.**

Expts. Fa and Fb.

Specific activity of oestrone isolated from incubation of hydatidiform mole (IV) with [7α-3H] testosterone (14 290 × 10^3 DPM; 250 µCi/5 µg).

<table>
<thead>
<tr>
<th>Crystals</th>
<th>Solvent</th>
<th>Fa</th>
<th>Fb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>^3H, DPM/mg</td>
<td>^3H, DPM/mg</td>
</tr>
<tr>
<td>first</td>
<td>acetone</td>
<td>473 300</td>
<td>590 500</td>
</tr>
<tr>
<td>second</td>
<td>methanol</td>
<td>449 800</td>
<td>580 100</td>
</tr>
<tr>
<td>third</td>
<td>ethanol</td>
<td>441 200</td>
<td>573 400</td>
</tr>
<tr>
<td>mother liquor</td>
<td></td>
<td>449 200</td>
<td>592 100</td>
</tr>
</tbody>
</table>

In expt. Fa 510 µg carrier oestrone added before initial extraction; 5200 µg standard oestrone added to 238 µg oestrone fraction from TLC.
In expt. Fb 1530 µg carrier oestrone added before initial extraction; 5200 µg standard oestrone added to 626 µg oestrone fraction from TLC.
This corrected percentage of conversion from \([7\alpha-^3\text{H}]\) testosterone into oestrone is 82.

\[ \text{Determination of endogenous production of oestradiol} \]

After TLC of the ether fraction in benzene:ethanol (9:1) and chloroform:acetone (8:2) standard oestradiol was added and the metabolite was crystallized to a constant specific activity (Table 12).

The endogenous production of oestradiol \((\text{XOE}_2)\) in experiment F can be calculated from the data presented in Table 12 in the same way as for oestrone.

Knowing the endogenous production of oestradiol \((\text{XOE}_2) = 31 \mu\text{g}\), the corrected percentage of conversion from \([7\alpha-^3\text{H}]\) testosterone into oestradiol can be calculated.

This corrected percentage of conversion from \([7\alpha-^3\text{H}]\) testosterone into oestradiol is 14.

\[ \text{Endogenous production of testosterone} \]

After TLC of the toluene fraction in chloroform:ethylacetate (8:2), benzene:ethanol (9:1), methylene chloride:acetone (8:2) and chloroform:acetone (8:2), the testosterone region was eluted, standard testosterone added and the radio-metabolite was crystallized to a constant specific activity (Table 13).

Calculated as described the endogenous production of testosterone \((\text{XT})\) is 65 \(\mu\text{g}\).

\[ \text{Table 12.} \]
Expts. Fa and Fb.
Specific activity of oestradiol isolated from incubation of hydatidiform mole (IV) with \([7\alpha-^3\text{H}]\) testosterone \((14\,290 \times 10^3 \text{ dpm}; 250 \mu\text{Ci}/5 \mu\text{g})\).

<table>
<thead>
<tr>
<th>Crystals</th>
<th>Solvent</th>
<th>Fa</th>
<th>Fb</th>
</tr>
</thead>
<tbody>
<tr>
<td>first</td>
<td>acetone</td>
<td>59 100</td>
<td>29 600</td>
</tr>
<tr>
<td>second</td>
<td>methanol</td>
<td>49 300</td>
<td>29 000</td>
</tr>
<tr>
<td>mother liquor</td>
<td></td>
<td>57 900</td>
<td>30 700</td>
</tr>
</tbody>
</table>

In expt. Fa 1506 \(\mu\text{g}\) carrier oestradiol added before initial extraction; 9900 \(\mu\text{g}\) standard oestradiol added to 490 \(\mu\text{g}\) oestradiol fraction from TLC.

In expt. Fb 500 \(\mu\text{g}\) carrier oestradiol added before initial extraction; 9900 \(\mu\text{g}\) standard oestradiol added to 80 \(\mu\text{g}\) oestradiol fraction from TLC.

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\(\text{Acta endocr. 64, 1}\)
Table 13.
Expts. Fa and Fb.
Specific activity of testosterone isolated from incubation of hydatidiform mole (IV) with [7α-3H] testosterone (14.290 X 10^3 DPM; 250 µCi/5 µg).

<table>
<thead>
<tr>
<th>Crystals</th>
<th>Solvent</th>
<th>Fa</th>
<th>Fb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3H, DPM/mg</td>
<td>3H, DPM/mg</td>
</tr>
<tr>
<td>first</td>
<td>acetone</td>
<td>4880</td>
<td>4540</td>
</tr>
<tr>
<td>second</td>
<td>methanol</td>
<td>4880</td>
<td>4530</td>
</tr>
<tr>
<td>mother liquor</td>
<td></td>
<td>4980</td>
<td>4550</td>
</tr>
</tbody>
</table>

In expt. Fa 494 µg carrier testosterone added before initial extraction; 10 140 µg standard testosterone added to 198 µg testosterone fraction from TLC. In expt. Fb 1482 µg carrier testosterone added before initial extraction; 10 140 µg standard testosterone added to 522 µg testosterone fraction from TLC.

Endogenous production of androstenedione

After TLC of the toluene fraction in chloroform:ethyl acetate (8:2), benzene: ethanol (9:1), methylene chloride:acetone (8:2) and chloroform:acetone (8:2), the androstenedione region was eluted, standard androstenedione added and

Table 14.
Expts. Fa and Fb.
Specific activity of androstenedione isolated from incubation of hydatidiform mole (IV) with [7α-3H] testosterone (14.290 X 10^3 DPM; 250 µCi/5 µg).

<table>
<thead>
<tr>
<th>Crystals</th>
<th>Solvent</th>
<th>Fa</th>
<th>Fb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3H, DPM/mg</td>
<td>3H, DPM/mg</td>
</tr>
<tr>
<td>first</td>
<td>acetone</td>
<td>3870</td>
<td>3440</td>
</tr>
<tr>
<td>second</td>
<td>methanol</td>
<td>3790</td>
<td>3400</td>
</tr>
<tr>
<td>mother liquor</td>
<td></td>
<td>3410</td>
<td></td>
</tr>
</tbody>
</table>

In expt. Fa 2084 µg carrier androstenedione added before initial extraction; 10 090 µg standard androstenedione added to 785 µg androstenedione fraction from TLC. In expt. Fb 500 µg carrier androstenedione added before initial extraction; 10 090 µg standard androstenedione added to 179 µg androstenedione fraction from TLC.
the radiometabolite was crystallized to a constant specific activity (Table 14). Knowing the endogenous production of androstenedione (XA is 85 µg), the correct percentage of conversion from [7α-3H] testosterone into androstenedione can be calculated.

This correct percentage of conversion from [7α-3H] testosterone into androstenedione is 0.8.

The values for the endogenous production of oestrone and oestradiol were thus extrapolated back to give approximate values for endogenous production during shorter time-intervals in the experiments D and E.

**DISCUSSION**

These experiments (A through F) provide evidence for metabolic conversion of testosterone and androstenedione into oestrone and oestradiol in hydatidiform mole tissue *in vitro*. There is no formation of oestriol from testosterone and androstenedione (experiments A, B, D and E). These data are in agreement with the findings that dehydroepiandrosterone is converted in molar tissue into oestrone and oestradiol, while information of oestriol does not occur (Van Leusden & Villee 1966a,b; Van Leusden 1966, 1967).

In experiment C equimolar amounts of testosterone and androstenedione were present at the start of the incubation. While this may not be «physiological» it makes interpretation of the data simpler. It is realized however, that substrate saturation curves would have been very useful.

Realizing these limitations it seems that the following evidence can be obtained from experiment C: as after 120 minutes incubation $^{14}$C/$^3$H in oestrone and oestradiol is 1.6 and remains 1.6 (Table 6) as compared with $^{14}$C/$^3$H 0.5 at the start of the experiment, androstenedione is a more effective precursor than testosterone for oestrone and oestradiol.

The same is found in experiments D and E (Tables 8 and 9) where the mass of testosterone at the start of the experiments is 44 times less than that of androstenedione: measured for oestrone after 5 min and for oestradiol after 10 min, androstenedione is a better precursor than testosterone.

Equilibrium oestrone $\rightleftharpoons$ oestradiol in experiment C (Table 6) apparently is established after 120 min as the ratio does not change and remains identical for oestrone and oestradiol. The data of Table 9 indicate that after 30 min equilibrium is reached. However, equilibrium androstenedione $\rightleftharpoons$ testosterone is not yet established after 120 min and not even after 240 min (Table 7). These data suggest that there are different $17\beta$-dehydrogenases for oestrone and androstenedione, but isolation and purification of these enzymes would be necessary to prove this assumption. Another possibility is that C-19 steric hindrance of $17\beta$-dehydrogenase plays a role. It seems rather unlikely that the
presence of large, though equimolar, amounts of androstenedione and testoste-
ron e at the start of the experiments could provide an explanation for the slower
establishment of androstenedione \(\rightarrow\) testosterone as compared with oestrone \(\rightarrow\)
oestradiol, as the findings of experiment C are in agreement with those of ex-
periments D and E where much lower amounts of androstenedione and testo-
sterone were present at the start of the experiments (Table 9).

\(^{14}\text{C}/{}^{3}\text{H}\) in oestrone after 5 min (Table 9) in experiments D and E is 17 times
that in androstenedione (Table 10). Thus under these experimental conditions
metabolic conversion of oestrone from androstenedione is much more effective
than conversion of testosterone into androstenedione (Table 10).

Of interest is the \(^{14}\text{C}/{}^{3}\text{H}\) in oestradiol after 5 min incubation (Table 9): 0.4
as compared with 0.6 at the start of the experiment. Apparently there is a
direct distribution of \([1,2-{}^{3}\text{H}]\) testosterone to oestradiol.

Comparing in the same experiment the percentual conversion of testosterone
into oestradiol and of testosterone into oestrone (Table 8) it is apparent that in
the 5 min incubation the percentage of conversion into oestradiol is twice that
into oestrone, which is also in agreement with hypothesis of a direct pathway
from testosterone to oestradiol prevailing in the shorter time incubations under
our experimental conditions. Thus, despite the fact that androstenedione is a
more effective precursor than testosterone for the formation of oestrone and
oestradiol (Table 8), there is a more effective contribution of testosterone to
oestradiol than to oestrone in the shorter period of incubation. The simplest
explanation for the generally decreasing nearly identical \(^{14}\text{C}/{}^{3}\text{H}\) in oestrone
and oestradiol after 30 min in experiments D and E (Table 9), is an indirect
contribution of testosterone to oestrone and oestradiol via androstenedione.

In the shorter time incubations with \([4-{}^{14}\text{C}]\) androstenedione and \([1,2-{}^{3}\text{H}]\)-
testosterone (experiments D and E) the percentages of conversion of oestrone
and oestradiol from androstenedione and testosterone show a gradual increase
with time (Table 8).

However, there is a dip in the percentual conversion of oestrone from andro-
stenedione in the 60 min incubation and there is – in contrast to oestrone – a
decrease in the percentual conversion of oestradiol from androstenedione as
well as from testosterone after 120 min. Endogenous production of steroids
during the incubations does not seem to be responsible for these findings, as
the same pattern is found after corrections for the endogenous production of
these steroids during the incubations (Table 8). We do not have an explanation
for these observations. During incubation in vitro of molar tissue, steroids are
synthetized and secreted regardless of whether exogenous precursors have been
added to the medium. If reverse isotope dilution analysis is to be accurate, the
contribution of the tissue to the total mass of each steroids isolated must either
be negligible or measured. The technique of using experiment F to correct for
endogenous production of steroids might be criticized on the grounds that ste-
roid production over a period of 240 min is unlikely to be linear. However, it is clear that the corrected percentages of conversion are substantial improvements on uncorrected ones. The endogenous productions of oestrone, oestradiol, testosterone and androstenedione determined in experiment F are 650, 31, 65 and 85 µg respectively/240 min. These findings indicate that oestrone is the most important steroid produced in vitro. The percentages of conversion of testosterone into oestrone, oestradiol and androstenedione in experiment F are 82, 14 and 0.8. One per cent of the radioactivity was recovered in testosterone after the incubation. Thus after 240 min metabolic conversion of testosterone into oestrone and oestradiol is almost complete.

REFERENCES


Cassmer O.: Acta endocr. (Kbh.) Suppl. 45 (1959) 32.


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