OESTROGEN METABOLISM

A Thesis presented for the Degree

of

Doctor of Philosophy

by

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May 1960

Contents

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SECTION I

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Oestrone is involved in this inactivation
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Oestradiol-17β

Oestriol

1. GENERAL INTRODUCTION

Although oestrone (Butenandt, 1929; Doisy,
Veler and Thayer, 1929), oestriol (Marrian,
1930) and oestradiol-17β (Wintersteiner, Schwenk
and Whitman, 1935) have been isolated from
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the suggestion was put forward that oestradiol-
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The liver is involved in this deactivation
process (see Mayer, 1952).

Oestrone   Oestradiol-17β

Oestriol
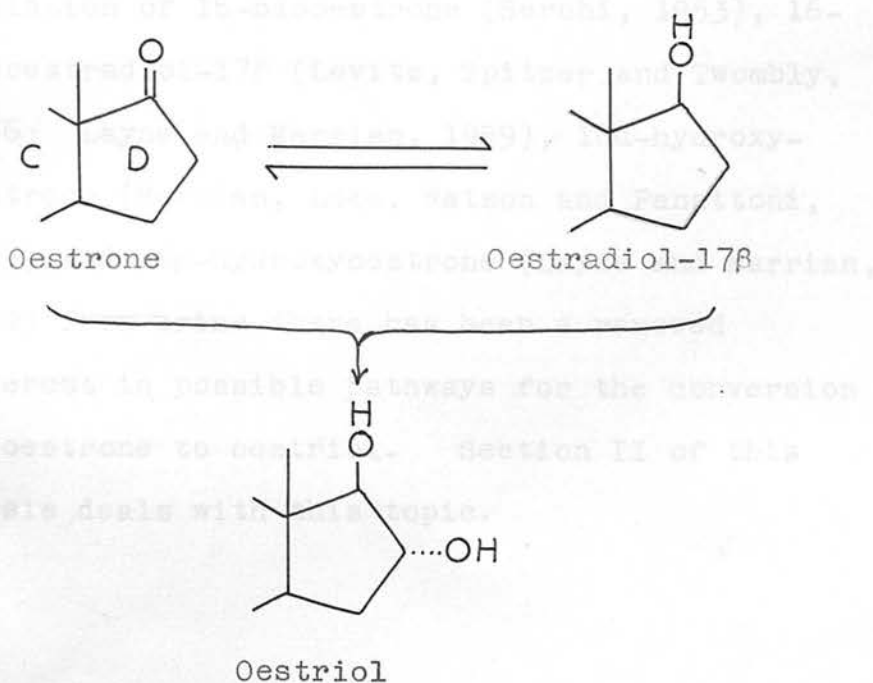
Numerous workers have shown that the oestrone-
oestradiol-17β interconversion can readily occur
in a number of species and tissues (see Lieberman
and Teich, 1953). Langer, Alexander and Engel
(1959) have shown that the purified oestradiol-
17β dehydrogenase from human placenta requires
either DPN$^+$ or TPN$^+$. Beer and Gallagher (1955)
have demonstrated that $\left[16^{14}C\right]$ oestradiol-17β or
$\left[16^{14}C\right]$ oestrone injected into women gives rise to
urinary $\left[^{14}C\right]$ oestriol. The reverse conversion
of oestriol to oestrone or oestradiol-17β does
not appear to occur (Pearlman, 1948). This has
been confirmed by Levitz, Spitzer and Twombly
(1958) who could detect no $\left[^{14}C\right]$ oestrone or
$\left[^{14}C\right]$ oestradiol-17β in the urine after injection
of $\left[16^{14}C\right]$ oestriol into women. With the
isolation of 16-oxooestrone (Serchi, 1953), 16-
oxooestradiol-17β (Levitz, Spitzer and Twombly,
1956; Layne and Marrian, 1959), 16α-hydroxy-
oestrone (Marrian, Loke, Watson and Panattoni,
1957) and 16β-hydroxyoestrone (Layne and Marrian,
1959) from urine there has been a renewed
interest in possible pathways for the conversion
of oestrone to oestriol. Section II of this
thesis deals with this topic.

Using a very elegant technique, Fishman, Bradlow and Gallagher (1959) have shown that it is oestrone rather than oestradiol-17β which undergoes further metabolic change.   They injected a mixture of 6,7 tritiated oestradiol-17β and $\left[16\ ^{14}C\right]$ oestrone into women and measured the ^{14}C/tritium ratios in the urinary oestrone, oestradiol-17β, oestriol, 16-<u>epi</u>oestriol and 2-methoxyoestrone at various time intervals after the injection.   The ratio of ^{14}C/tritium was high in all the compounds mentioned.

The only evidence that oestradiol-17β is the primary oestrogenic hormone comes from experiments in which radioactive oestrone and oestradiol-17β have been synthesized from precursors such as $\left[^{14}C\right]$ acetate.   Thus Rabinowitz and Dowben (1955) found that dog ovary slices or homogenates could synthesize $\left[^{14}C\right]$ oestradiol-17β and $\left[^{14}C\right]$ oestrone from $\left[2\ ^{14}C\right]$ acetate.   In all the experiments they performed, the specific activity of the oestradiol-17β was greater than that of the oestrone.   On the precursor-product relation-ship this would indicate that oestradiol-17β was the first formed compound.

Largely due to the use of isotopically labelled oestrogens, a large number of papers on the chemistry, metabolism and mode of action of oestrogens have appeared in recent years. Thus the number of known naturally occurring oestrogens has trebled in the last five years.

Until recently, investigations in the oestrogen field have been concerned either with the metabolism of these compounds or with their biological action.   In 1958, Talalay and Williams-Ashman put forward the suggestion that these two aspects of oestrogen biochemistry might, in some cases, be one and the same thing. They put forward evidence that human placental oestradiol-17β dehydrogenase also catalysed a DPN-TPN transhydrogenation.   To explain this they postulated that the following was occurring:-

DPNH ⟵   ⟶ OESTRONE ⟵   ⟶ TPNH

DPN⁺ ⟵   ⟶ OESTRADIOL-17β ⟵   ⟶ TPN⁺
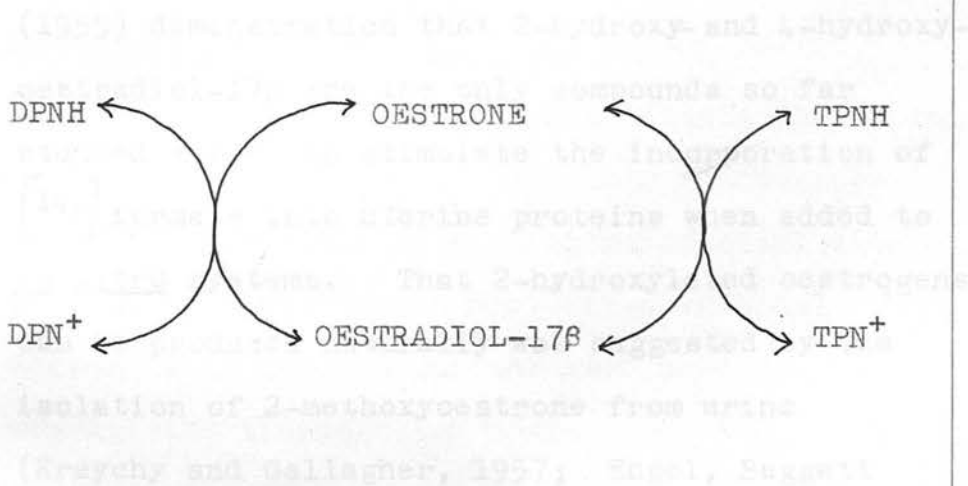
They claim that the transhydrogenase and the dehydrogenase are the same enzyme and that the oestrogen acts as a cofactor.   Villee and co-workers have questioned this (see Hagerman and Villee, 1958) and say that the oestrogen activates an inactive form of the trans-hydrogenase.   Furthermore, they claim to have separated the dehydrogenase and transhydrogenase activities.

Although oestrone and oestradiol-17β can stimulate this transhydrogenase, potent in vivo oestrogens like stilboestrol and oestriol are inactive.   To account for this Williams-Ashman, Cassman and Klavins (1959) have suggested that these compounds might exert their biological action via the corresponding 2- or 4-hydroxy compound.   This would fit in with Mueller's (1955) demonstration that 2-hydroxy-and 4-hydroxy-oestradiol-17β are the only compounds so far studied which can stimulate the incorporation of $\left[^{14}C\right]$ formate into uterine proteins when added to in vitro systems.   That 2-hydroxylated oestrogens can be produced naturally was suggested by the isolation of 2-methoxyoestrone from urine (Kraychy and Gallagher, 1957;   Engel, Baggett

and Carter, 1957; Loke and Marrian, 1958).   No
2-hydroxy compounds have yet been isolated from
urine.   The in vitro synthesis of 2-hydroxy and
2-methoxy oestrogens is described in Section III
of this thesis.

2.   EXPERIMENTAL

a.   Preparation of Tissue Slices

The rats were killed by breaking their necks.
The necessary organs were immediately removed and
kept on ice until used.   This was usually not
longer than 10 min.   Slices were cut free-hand
with a razor blade so that the slices were about
0.5 mm. thick.   The slices were washed in ice-
cold saline and blotted dry on filter paper
before weighing.

b.   Preparation of Homogenates

The tissue was minced in a Latapie mincer to
remove connective tissue, and then homogenized
with 0.25 M-sucrose in a glass homogeniser with
a nylon pestle.   Routinely, seven vertical
movements of the homogeniser were used to
disrupt the tissue.

c. Tissue Fractions

The preparation of cell fractions was based
on the method of Hogeboom (1955). The
homogenized tissue (see b. above) was centrifuged
for 10 min. at 700 g to remove erythrocytes,
residual intact cells and nuclei. By this
method about 10% of the free mitochondria are
sedimented with this fraction but, as no
quantitative experiments were carried out with
the mitochondrial fraction, this was not
important.

The mitochondria were sedimented by a 10
min. centrifugation at 7,000 g. As far as was
possible, the 'fluffy layer' obtained from liver
homogenates was left with the mitochondrial
fraction. The mitochondria were not further
purified.

The microsomes were separated from the
supernatant by centrifuging for 30 min. at
105,000 g in a preparative Spinco model L
centrifuge. In the experiments mentioned in
Appendix II, 5, the microsomes were isolated by
centrifuging the microsome plus supernatant
fraction for 2 hr. at 36,000 g in an M.S.E.
refrigerated centrifuge with high speed

attachment.

d. Addition of Steroids

All steroids used were dissolved in propylene glycol (PG) and added to the incubation tubes from a micrometer syringe.

e. Preparation of Buffers

i. Krebs-Ringer phosphate saline. This was prepared according to Umbreit, Burris and Stauffer (1949). It was made up fresh from its component solutions for every experiment. The sodium phosphate buffer was prepared by dissolving the required amount of sodium phosphate in water and then adding 15% (v/v) HCl dropwise till the pH reached 7.4 (as measured with a pH meter). The solution was then made up to the required volume.

ii. Potassium phosphate buffer. The same procedure as was used for the sodium phosphate buffer was employed except that potassium dihydrogen phosphate was used and the pH was adjusted to 7.4 with 3 N-KOH.

f. Gassing of Solutions and Incubation Flasks

The required gas was always bubbled through the saline or buffer to be used for 30 min. prior to incubation. The pH of the solution

was checked just before setting up the incubation
flasks.   In the experiments in which air was not
the gas phase, the required gas was played on the
surface of the incubation mixture for 30 sec. and
then the flasks were immediately stoppered.   In
the large scale incubation experiments this
gassing period was extended to 1 min.

g.   Kober Reaction

The Kober reaction as modified by Brown
(1952) and Bauld (1954) was used.   In all cases
Bauld's 'oestriol' reagent was used.   The
reaction was carried out as follows:- 50 mg. of
hydroquinone were added to each estimation tube
containing the dry oestrogen residue and 2.6 ml.
of Kober reagent were added and heated in a
vigorously boiling water bath for 20 min. with
frequent shaking.   The tubes were then cooled
in water and a further 50 mg. of hydroquinone
added, followed by 0.7 ml. of water.   The
contents were well mixed and reheated for another
15 min.   After cooling, the optical densities
were read in 1 cm. light path glass cells at
480 mμ, 512.5 mμ and 545 mμ.   By applying the
Allen correction (Allen, 1950), the corrected
optical density at 512.5 mμ could be obtained.

This is achieved by subtracting the average of the readings at 480 mμ and 545 mμ from that at 512.5 mμ. The absorption maximum of the typical Kober colour is 512.5 mμ.

The use of the Allen correction involves two assumptions.

 i. The Kober colour absorption curve must be symmetrical about the maximum.

 ii. The interfering background colour must have a linear absorption curve over the wavelengths used.

Assumption i. holds for oestriol and ii. holds for urinary extracts (Diczfalusy, 1955), but its use with tissue extracts has not been conclusively justified. The only evidence that its use under these conditions is permissible is the zero readings obtained with liver tissue in the absence of steroid and the fairly constant recoveries of oestriol added to varying amounts of tissue.

h. Partition Column Chromatography

Celite columns were prepared by the method of Bauld (1955). The solvent system used was 70 methanol 30 water/ethylene dichloride (hereafter referred to as 70% methanol/ethylene dichloride). The two phases were equilibrated

for at least 1 hr. before use.   A mixture of
stationary phase and celite was prepared by
thoroughly mixing equal amounts of the two (v/w).
Sufficient mobile phase was then added to
prepare a slurry which could be packed quite
readily.   Unless otherwise stated, columns were
1 x 10 cm.   They were packed to run at about
15 ml./hr.   In all cases the running on volume
was discarded.

In this system 16-_epi_oestriol and 17-_epi_-
oestriol are eluted with $2 \rightarrow 8$ ml. of ethylene
dichloride, and oestriol with $10 \rightarrow 35$ ml. of this
solvent.

i.   Paper Chromatography

This was carried out in a thermostatically
controlled room at $20° \pm 2°C$.

i.   Formamide/chloroform system.   Whatman
no. 42 paper was used.   This was impregnated
with the stationary phase by dipping the paper
through a trough containing 2 vol. methanol:
1 vol. formamide.   The excess fluid was removed
by laying the paper on a glass sheet, covering
this with a larger piece of Whatman no. 42 paper
and then pressing on top of this with another
glass sheet.   The impregnated paper then stood

at 37°C. for 45 min. to remove the methanol.
The material to be chromatographed, usually
dissolved in methanol, was then spotted onto the
paper and the paper allowed to stand in the tank
for at least 15 min. before adding the mobile
phase.

ii. Other systems. In all the systems in
which formamide was not the stationary phase, the
paper was allowed to equilibrate in the tank
overnight before development. Throughout the
thesis the term 70% acetic acid will refer to
70% acetic acid:30% water.

j. Location of Oestrogens on Chromatograms

Unless otherwise stated, the oestrogens
were detected by spraying the paper with a 4:1
mixture of water and Folin and Ciocalteu reagent
(Davies and Mitchell, 1954). The paper was
then suspended in an atmosphere of ammonia to
develop the blue colour characteristic of phenols.
It should be borne in mind that when this method
is used it is phenols, not necessarily oestrogens,
which are being detected.

k. Girard Reaction

The material was dissolved in 1 ml. of
ethanol + 1 ml. of glacial acetic acid.

Girard reagent T (hydrazino-carbonyl-methyl
trimethylammonium chloride) (5-10 mg.) was added
and the solution allowed to stand overnight at
room temperature.   The vessel was then chilled
in the deep-freeze and 90% neutralized with
cold N-NaOH.   The NaOH had been standardized
previously against the acetic acid.   About 2 g.
of sodium chloride were added to prevent
emulsions on subsequent extraction and the
volume made up to 25 ml. with ice-cold water.
This was extracted three times with 25 ml. of
ether and the combined ether extracts washed
once with 5 ml. of water and the water washings
added to the aqueous phase.   The ether (non-
ketonic fraction) was then further washed twice
with 5 ml. of 5% NaHCO$_3$ and once with 5 ml. of
water.   The ether was taken to dryness under
vacuum.

The aqueous phase was acidified with 6 ml.
of conc. HCl and left at room temperature for 1
hr. to hydrolyse the Girard complex.   The
solution was then extracted three times with 25
ml. of ether and the ether (ketonic fraction)
washed twice with 5 ml. of 5% NaHCO$_3$ and once
with 5 ml. of water.   The ether was taken to

dryness under vacuum.

1. Sodium Borohydride Reduction

    The material to be reduced was dissolved in
1 ml. of methanol and 2 mg. of sodium borohydride
added.   The solution stood for 1 hr. at room
temperature and was then diluted with 10 ml. of
15% (v/v) HCl plus 39 ml. of water.   The
resultant mixture was extracted three times with
50 ml. of ether and the ether washed twice with
20 ml. of water.   The ether was taken to dryness
under vacuum.

m. Fast Black Salt K Formation

    These derivatives were prepared by the
method of Heftmann (1950).   About 15 μ g. of the
oestrogen in 0.1 ml. of methanol, 0.1 ml. of
20% Na_2CO_3 and 0.2 ml. of a fresh, saturated
aqueous solution of Fast Black Salt K
(diazotized p-nitrophenyl azo dimethoxy aniline)
were heated in a boiling water bath for 10 min.
After cooling, the derivatives were extracted
once with 0.2 ml. of benzene and the benzene
solution spotted directly onto Whatman no. 1
paper and chromatographed for 2 hr. in the
system 30 ethanol:70 water/2 toluene:1 pet. ether
(40-60°).

n. <u>Melting Point Determinations</u>

Unless otherwise stated, these were carried
out in sealed evacuated tubes.   They are
uncorrected for emergent stem.

o. <u>Experimental Results</u>

Unless stated otherwise, each incubation
was done in duplicate and each experiment
carried out at least twice so that each result
represents not less than four estimations.

1. INTRODUCTION

A number of pathways have been suggested for the conversion of oestradiol-17β and oestrone to oestriol. Marrian (1939) suggested that oestradiol-17β might be dehydrogenated between carbon atoms 16 and 17 to produce the enol form of oestrone, which, on subsequent hydration could produce oestriol.

<p style="text-align:center"><u>SECTION II</u></p>
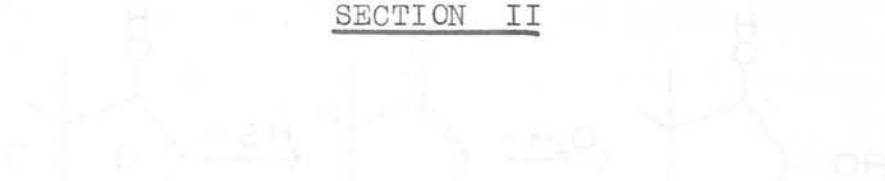
<u>IN VITRO METABOLISM OF 16α-HYDROXYOESTRONE,</u>

<u>16-OXOOESTRADIOL-17β AND 16-OXOOESTRONE</u>

However, the enol forms of 17-ketosteroids are not very stable and this suggestion has received very little further attention.

In 1959, Hoffman and Grollman put forward the following suggestion, based on the relative activities of a number of compounds which they had tested.

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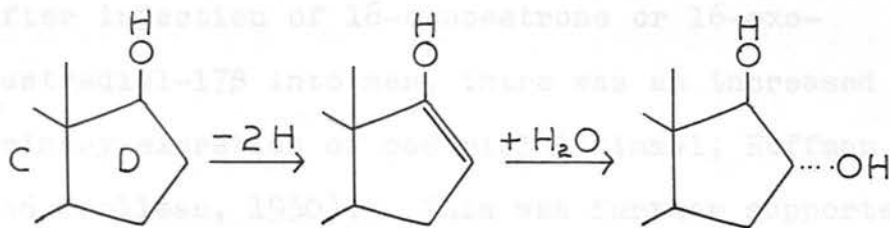
Oestradiol-17β   Enol form of   Oestriol
                     oestrone

However, the enol forms of 17-ketosteroids are not very stable and this suggestion has received very little further attention.

In 1947, Huffman and Grollman put forward the following suggestion, based on the relative bioactivities of a number of compounds which they had tested.

Oestrone   16-Oxooestrone   16-Oxo-
                                      oestradiol-17β

Oestriol

They produced evidence for such a pathway when,
after injection of 16-oxooestrone or 16-oxo-
oestradiol-17β into men, there was an increased
urinary excretion of oestriol (Stimmel, Huffman
and Grollman, 1950).   This was further supported
by the isolation of 16-oxooestrone from female
urine by Serchi (1953).   This evidence should,
however, be treated with some reserve as he
claims to have crystallized the material from 10
litres of menstrual cycle urine.   If 16-oxo-
oestrone is present in such amounts, it seems
strange that it has not been isolated by other
workers.   The demonstration by Levitz, Spitzer
and Twombly (1956) that $[16^{14}C]$ oestradiol-17β is
in part excreted in the urine as $[^{14}C]$ 16-oxo-
oestradiol-17β also fits in with this biogenetic
scheme.   Both of the pathways mentioned above

could account for the production of 16-epi-
oestriol which has been isolated from pregnancy
urine by Marrian and Bauld (1955).

In all cases so far investigated in the
steroid field, an oxo group is produced by
oxidation of a hydroxyl group and there does not
appear to be any direct introduction of a
carbonyl oxygen into the molecule. So, if 16-
oxooestrone is formed directly from oestrone it
is a unique type of biochemical reaction in the
steroid field.

When Marrian, Loke, Watson and Panattoni
(1957) isolated 16α-hydroxyoestrone from
pregnancy urine, a new pathway was suggested.

Oestrone   16α-Hydroxy-   Oestriol
                   oestrone

They also suggested that 16-epioestriol could
arise in a similar way from 16β-hydroxyoestrone.
That this pathway probably does occur was
supported by the isolation of 16β-hydroxyoestrone
from pregnancy urine (Layne and Marrian, 1958)
and as a urinary metabolite of $[16^{14}C]$ oestradiol-
17β (Brown, Fishman and Gallagher, 1958).

Both Marrian's hypothesis (1957) and
Huffman's scheme fit in with the view that it is
oestrone rather than oestradiol-17β which under-
goes further metabolic change (Fishman, Bradlow
and Gallagher, 1959).

In order to find out which of the two main
suggested pathways is the functional one it was
decided to investigate the in vitro metabolism of
16α-hydroxyoestrone, 16-oxooestradiol-17β and 16-
oxooestrone.

Levitz, Spitzer and Twombly (1958) have
shown that $[16^{14}C]$ oestriol can be converted to
16-oxooestradiol-17β and 16-epioestriol in vivo
and it was thus of interest to see if this
reaction could be duplicated in vitro.

It is well established that the liver is
concerned in the biological deactivation of
oestrogens and Mayer (1952) has demonstrated that

in dogs the conversion of oestradiol-17β to oestriol occurs mainly in the liver. This suggested that the liver would be a suitable organ to use in these experiments. The rat was chosen as a convenient experimental animal.

2. EXPERIMENTAL

All the methods used in this section are described in the General Experimental Methods Section (I, 2).

3. DEVELOPMENT OF A METHOD FOR THE EXTRACTION OF OESTRIOL FROM TISSUE INCUBATIONS

a. Preliminary

It was decided to adapt the extraction procedure used for urinary oestrogens (Cohen and Marrian, 1934). In essential outline this consists of acid hydrolysis of urinary conjugates followed by ether extraction of the free oestrogens. The ether is washed with

bicarbonate to remove acid material and then shaken with sodium hydroxide to extract any phenols. The alkaline extract is then neutralized, re-extracted with ether and, after washing with water, the ether is taken to dryness.

Alpha ketols such as 16α-hydroxyoestrone, 16β-hydroxyoestrone and 16-oxooestradiol-17β are unstable in alkali (Loke, 1958) and consequently the above extraction procedure would seriously interfere with the estimation of any of these compounds. It was proposed to use Bauld's method (1955) to separate the oestrogens after extraction, but in this method compounds such as 16α-hydroxyoestrone are not separated from 16-epi-oestriol and would thus interfere with the estimation of the latter compound. In order to remove these difficulties, it was decided to let the extracted oestrogens stand in strong alkali overnight. This completely destroys any 16α-hydroxyoestrone and 16-oxooestradiol-17β which might be present (Loke, 1958).

Several workers have separated the tissue slices and incubation medium prior to extraction (Sandberg, Slaunwhite and Antoniades, 1957; Jellinck, 1959) and then treated the two portions

separately.   Sandberg, Slaunwhite and Antoniades
go so far as to call the material extracted from
the separated slices 'protein bound'.   They
apparently ignore the possibility that there are
any free oestrogens inside the liver slices.
That the latter may well be the case is suggested
by the experiments of Jellinck (1959).   When he
incubated boiled rat liver slices with $\left[16^{14}C\right]$ -
oestrone and separated the slices and medium,
only 25% of the $\left[^{14}C\right]$ could be extracted from the
medium with ether.   A further 50% was recovered
in the ethanolic extract of the liver.   After
incubation in the absence of tissue, 79% of the
radioactivity was ether extractable.

In all the experiments described in this
thesis, tissue and medium were extracted together.

b.   Experiments on a Hydrolysis Step to
     Hydrolyse any Conjugates

Since Crépy (1947) has shown that rabbit and
guinea-pig liver slices can produce oestriol
glucuronide, it was decided to include a hydrolysis
step in the extraction procedure.

In an attempt to break up tissue slices,
hydrolyse conjugates and destroy the α-ketols in

one step, the effect of standing oestriol in 20%
KOH in the presence and absence of tissue was
tested.   When allowed to stand overnight at 37°C,
the tissue was completely disrupted, but only
about 30% of the added oestriol could be recovered.
If no liver was present, the recovery was about
80%.   The nature of this loss was not
investigated further.   Hecker and Mueller (1958)
have shown that boiled rat liver microsomes can
bring about protein binding of $\left[8^{14}C\right]$ tetrahydro-
2-naphthol.

HO
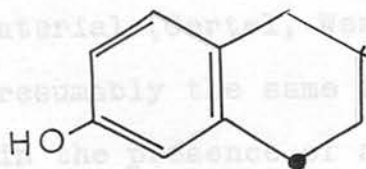
This compound is similar to the A and B rings of
oestriol, and a similar binding of the oestriol
may be occurring here.   Another possibility is
that the oestriol has become esterified to free
carboxyl groups of amino acids or peptides
formed by hydrolysis of the tissue proteins.

Acid hydrolysis was then tested.   The
tissue slices and incubation medium were
homogenized in a glass homogeniser, 10 ml. of

15% (v/v) HCl added and the mixture heated in a
boiling water bath for 30 min.   When the
hydrolysis was carried out in the absence of
tissue, about 70% of the added oestriol was
recovered.   Similar recoveries were obtained
when the acid was added in the presence of tissue
but the 30 min. hydrolysis omitted.   When the
hydrolysis was carried out in the presence of
tissue, only 30% of the oestriol could be
recovered.   This was unexpected as blood may be
processed in a similar way without any apparent
loss of material (Oertel, West and Eik-Nes, 1959).
This is presumably the same type of loss as
occurred in the presence of alkali.

In view of these results, acid was added to
the homogenized incubation medium but no
hydrolysis was performed.   Consequently this
method does not estimate any conjugated oestrogens.
Later results indicated that there were no
conjugates formed (Section III, 13).

c.   Final Extraction Procedure

    i.   Incubation medium and tissue homogenized.
    ii.   15 ml. of 15% (v/v) HCl added.
Extracted three times with 50 ml. of ether.

iii. Ether washed twice with 20 ml. of 5%
(w/v) NaHCO$_3$.

iv. Ether extracted three times with 20 ml.
of N-NaOH. Sodium chloride (1 g.) was added
before each extraction to prevent the formation
of emulsions. The alkaline extract stood at
37°C overnight.

v. The alkali was partly neutralized with
10 ml. of 5 N-H$_2$SO$_4$ and then brought to pH 8-10
by bubbling carbon dioxide through the solution.
The pH was checked with phenol phthalein
indicator paper.

vi. Sodium chloride (10 g.) was added to
prevent emulsions and the mixture extracted
three times with 50 ml. of ether.

vii. The ether was washed twice with 20 ml.
of water and then taken to dryness under vacuum.

d. Separation and Estimation of Oestriol and
16-epiOestriol

The dry residue was dissolved in 2.5 ml. of
ethylene dichloride and a 2 ml. portion
chromatographed on a celite column (see
Experimental Section h). The required eluates
were taken to dryness in a hot water bath with

the aid of an air blast.   The oestrogen content
of the dry residue was estimated by the Kober
reaction (Section I, 2, g).

4.   METABOLISM OF 16α-HYDROXYOESTRONE BY RAT LIVER SLICES

a. Preliminary Experiments

Male rats were used initially to eliminate
possible variations in activity due to the
oestrus cycle and also to reduce interference
from endogenous oestrogens in the tissue.   As
the conversion of 16α-hydroxyoestrone to oestriol
occurred readily with male liver slices, adult
male rats were used for all the experiments.

The results of the first experiment are
given below.   Each incubation tube contained
2 ml. of Krebs Ringer phosphate pH 7.4 plus
200 mg. wet weight liver slices.   Incubations
were done in air for 2 hr. at 37°C.

FIG.1. ELUTION PATTERN OF 16ɑ-OH OESTRONE METABOLITES FROM CELITE COLUMN WITH THE SYSTEM 70 METHANOL : 30 WATER / ETHYLENE DICHLORIDE.
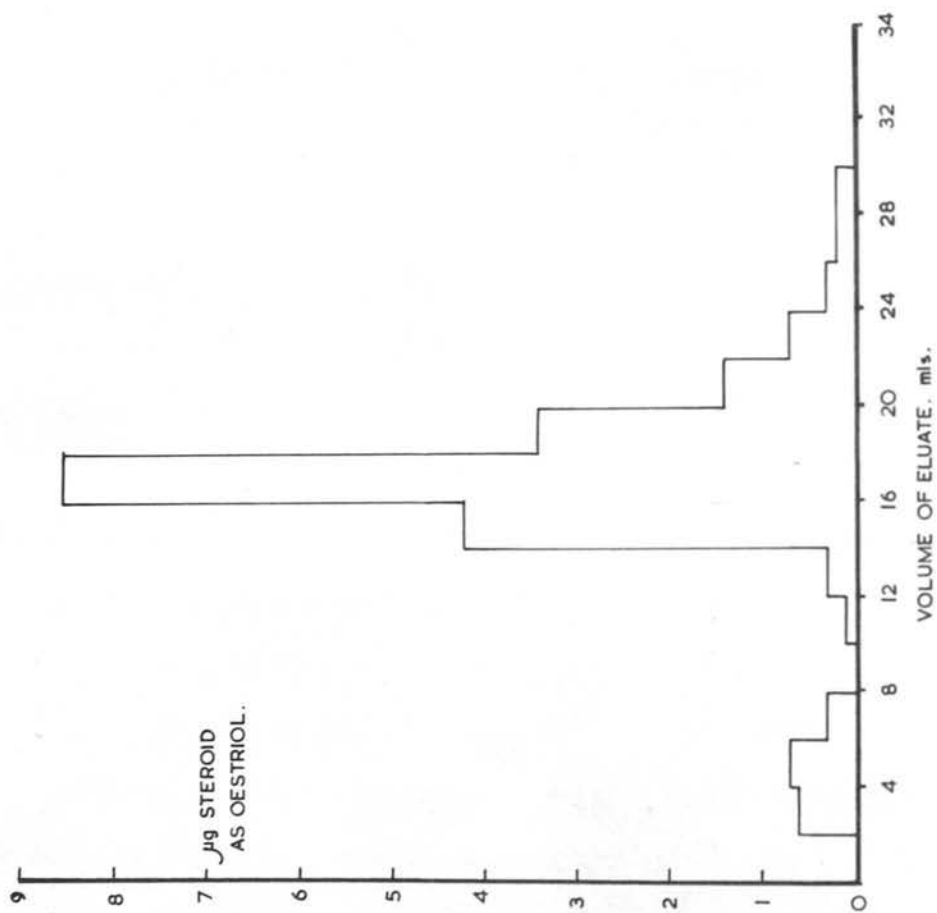
Steroid Added	Oestriol Recovered %
None	0
100 μg. oestriol in 0.05 ml. PG added after the incubation	69
100 μg. oestriol in 0.05 ml. PG added before the incubation	32
100 μg. 16α-hydroxy-oestrone in 0.05 ml. PG added before the incubation	14

The low recovery of oestriol after incubation with liver slices suggested that the yield of oestriol from 16α-hydroxyoestrone was appreciably higher than the 14% shown here.

In one of the 16α-hydroxyoestrone incubations, 2 ml. cuts were collected from the celite column and each cut estimated separately. The elution pattern is shown in Fig. 1.   The material eluted with 12 → 30 ml. of ethylene dichloride had the same elution pattern as

FIG.2. CONVERSION OF 16⍺-OH OESTRONE TO OESTRIOL BY RAT LIVER SLICES.

●------● INCUBATED UNDER NITROGEN.

○————○ INCUBATED UNDER AIR.
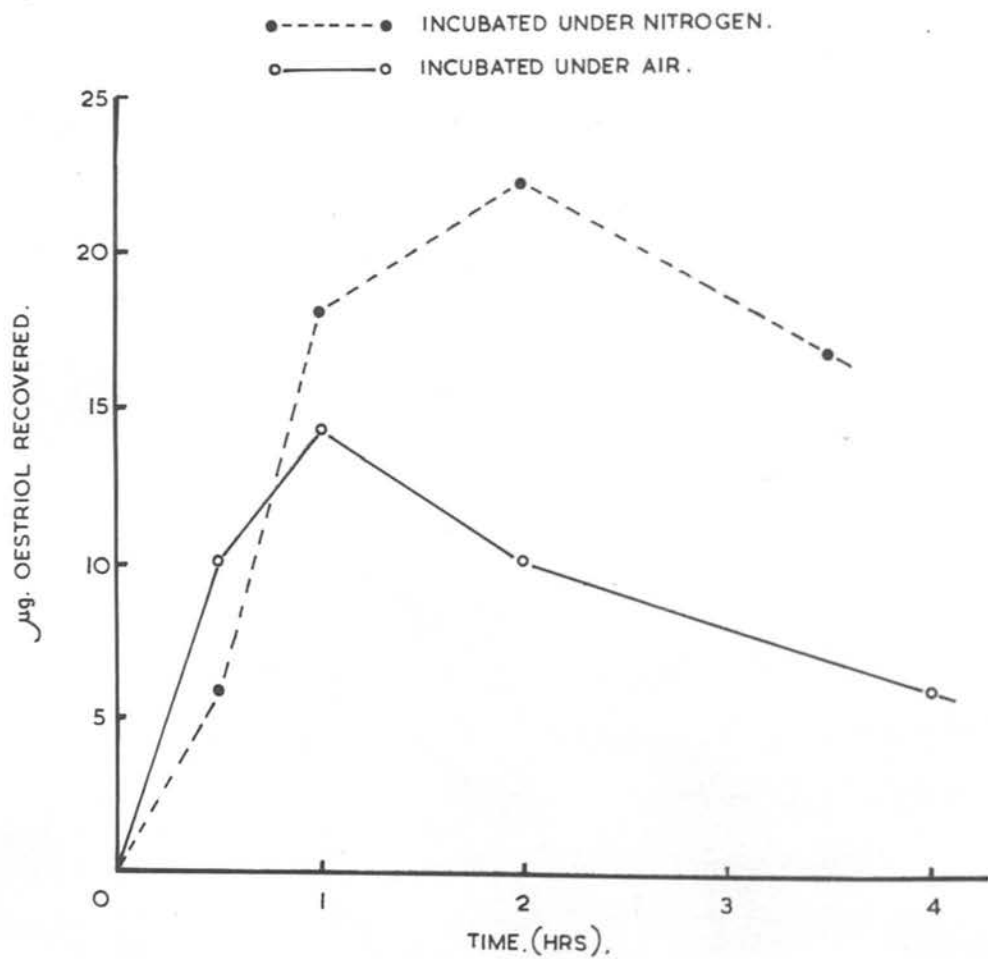
200 mg. RAT LIVER SLICES + 2 ml. KREBS-RINGER PHOSPHATE pH 7·4 + 100 μg. 16⍺-OH OESTRONE IN 0·05 ml. PG.   INCUBATED AT 37°C.

oestriol and behaved in the same way as oestriol
in the Kober reaction.   Its identity with
oestriol was proved by later large scale
incubations.

The Kober chromogenic material eluted with
$2 \rightarrow 8$ ml. is of some interest.   The most likely
second metabolite of 16α-hydroxyoestrone is 17-epi-
oestriol.   17-epiOestriol would be eluted in
this fraction.   If the phenolic fraction of the
incubation is chromatographed in the formamide/
chloroform system for 10 hr. there is a trace of
a phenol with the same mobility as 17-epioestriol.
There is no trace of any α-ketols or 16-epi-
oestriol.   Assuming that 17-epioestriol gives
the same intensity as oestriol in the Kober
reaction, then there is about 1% conversion of
16α-hydroxyoestrone to 17-epioestriol.

b.   Effect of Varying the Incubation Time

This is shown in Fig. 2.   Apart from the
incubation time, conditions were the same as in
the preliminary experiment described above.
The simplest explanation of the lower yield of
oestriol after longer incubation time was that
the oestriol, once formed, was itself metabolized
to unknown products.

FIG. 3.

EFFECT OF DIFFERENT MIXTURES OF OXYGEN AND NITROGEN
ON OESTRIOL METABOLISM BY RAT LIVER SLICES.
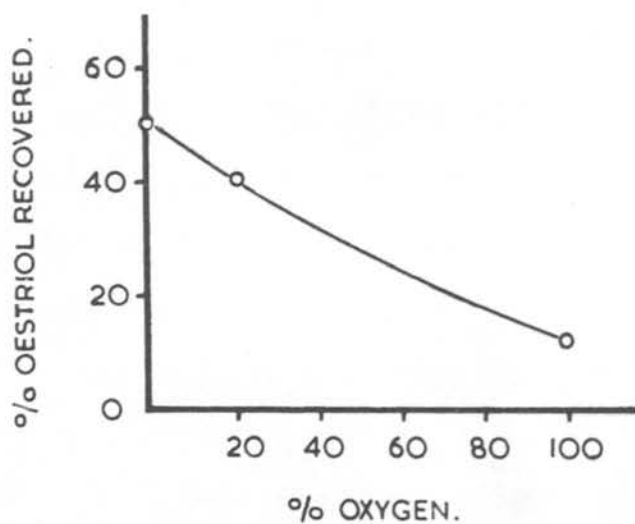
200mg. RAT LIVER SLICES + 2ml. KREBS-RINGER PHOSPHATE pH 7·4
+ 100 μg. OESTRIOL IN 0·05ml. PG. INCUBATED 1 HR. 37°C.

c. Effect of Varying the Gas Phase

A number of workers (Ryan and Engel, 1953;
Szego, 1953; Reigel and Mueller, 1954) have
shown that the in vitro metabolism of oestrogens
is markedly influenced by the gas phase above
the incubation medium. It was thus decided to
check the effect of the gas phase on the amount
of oestriol metabolized. The amount of oestriol
recovered after incubating 100 μg. of oestriol
with 200 mg. of liver slices in 2 ml. of Krebs-
Ringer phosphate, pH 7.4, under different gas
phases is shown in Fig. 3. During the 1 hr.
incubation, the appropriate gas was slowly
bubbled through the incubation medium. The
nitrogen used was unpurified commercial material
and probably contained 1-2% of oxygen. This,
plus the fact that the slices were not pre-
incubated before the addition of oestriol
suggests that the amount of oestriol metabolized
under completely anaerobic conditions is
probably even less than shown here. In the
extraction control, 70% of the added oestriol was
recovered.

This result thus agrees with previous
workers and poses some interesting questions,

some of which are answered in Section III.
The implications of this oxygen dependence are
discussed in that Section.

The time course experiment was then
repeated using nitrogen as the gas phase.   The
result is shown in Fig. 2.   As expected, the
yield of oestriol was improved.   The fall after
3.5 hr. may be due to the presence of small
amounts of oxygen.

d.   Effect of Varying the Amount of Tissue

The results are given below.   Incubations
were for 2 hr. under nitrogen.

Wet weight of tissue mg.	Conversion to oestriol %	Wet weight of tissue mg.	Conversion to oestriol %
100	20	300	25
200	23	400	14

The lower yield of oestriol with 400 mg. of
tissue is presumably due to the complex
metabolism of oestriol and possibly 16α-hydroxy
oestrone (Section III).

e. Characterization of the Metabolically Formed
   Oestriol

To make sure that it was oestriol we were
measuring, 32 mg. of 16α-hydroxyoestrone were
incubated under nitrogen for 2 hr. with the same
steroid:tissue:buffer ratio as was used in the
preliminary experiment (a. above). The
extraction was as before except that it was
scaled up three times.

This gave a dark oil which was appreciably
cleaned up by alumina chromatography. Alumina
(10 g.), acid washed, activated at 140°C and then
deactivated with 5% of water, was made up into a
1.5 x 8 cm. column in benzene. Discontinuous
gradient elution with increasing amounts of
methanol in benzene was used. In all cases the
oil was leached with solvent before the latter was
transferred to the column. Successive 10 ml.
cuts were collected. Nothing was eluted with
50 ml. each of 1% methanol or 2% methanol in
benzene. After the first 50 ml. of 5% methanol
in benzene, material was eluted which, on
evaporation gave a semi-crystalline oil. This
was all eluted with 100 ml. of this solvent.

The eluting solvent was then changed to 10%
methanol in benzene, but no more Kober positive
material was eluted.

The 5% methanol in benzene fractions were
combined and dried in vacuo to give 60 mg. of a
pale brown oil.   The Kober chromogen content of
this was about 3.6 mg. of oestriol.   The oil
was refluxed for 15 min. with 5% KOH in ethanol.
This was diluted with 50 ml. of water, acidified
with 5 N-H_2SO_4, and extracted three times with
100 ml. of ether.   The ether extract was washed
once with 50 ml. of 5% (w/v) $NaHCO_3$ and three
times with 50 ml. of water.   The ether was taken
to dryness to give 49 mg. of oil.

An appreciable amount of the pigment present
in this fraction was removed by leaching five
times with 1 ml. portions of warm benzene.   The
remaining whitish solid (3 mg.) was acetylated
with 0.2 ml. of acetic anhydride plus 0.2 ml. of
pyridine overnight at room temperature.   The
excess acetic anhydride was decomposed with
crushed ice and the solution taken to dryness in
vacuo.   The white residue was crystallized from
0.5 ml. of warm n-hexane, and the crystals
washed twice with 1 ml. portions of n-hexane.

The product gave m.p. 122-124°C with softening

at 121°C (cf. authentic oestriol triacetate

m.p. 125-127°C).   The mixed m.p. with authentic

oestriol triacetate was 123-126°C.

A blank incubation was carried out in which

the same amount of tissue and buffer was used,

but no 16α-hydroxyoestrone was added.   This was

processed as above until after the alumina step.

One-fifth of this residue contained no Kober

chromogenic material.

This conclusively shows that the 16α-hydroxy-

oestrone is being converted to oestriol.

5.   EXPERIMENTS WITH 16-OXOOESTRADIOL-17β AND

16-OXOOESTRONE

16-Oxooestradiol-17β (100 μg.) or 16-oxo-

oestrone (100 μg.) was incubated with 200 mg. of

slices plus 2 ml. of Krebs-Ringer phosphate, pH

7.4, for 2 hr. under nitrogen.

Steroid Incubated	Oestriol Formed %	16-epiOestriol Formed %
16-Oxooestradiol-17β	2	17
16-Oxooestrone	6	16

The material estimated as 16-epioestriol had the
same mobility as 16-epioestriol in the formamide/
chloroform system, and had the same elution
pattern as 16-epioestriol from celite columns in
the system 70% methanol/ethylene dichloride.
It also behaved in the same way as 16-epioestriol
in the Kober reaction (Bauld, 1955). These
estimations are worked out on the assumption that
16-epioestriol gives the same intensity of Kober
colour as oestriol (weight for weight). Bauld
(1955) has shown that at the 5 µg. level,
16-epioestriol gives 104% of the colour of
oestriol.

6. <u>WORKING UP OF 'ELUATE FRACTION' FROM
THE LARGE SCALE OESTRIOL METABOLISM
EXPERIMENTS</u> (Section III, 4)

If any compounds less polar than 17-<u>epi</u>-
oestriol had been formed from oestriol, they
would be present in this fraction. The eluate
fraction from 240 mg. of oestriol was
chromatographed on a 1 x 10 cm. celite column
with the system 70% methanol/ethylene dichloride.
Discarding the running-on volume, four 2 ml. cuts
and one 10 ml. cut were collected. Each cut was
taken to dryness under nitrogen, and one-fifth of
each cut chromatographed on paper in the
formamide/chloroform system for 10 hr. 16-Oxo-
oestradiol-17β and 16α-hydroxyoestrone were run
as standards. No α-ketols could be detected in
any of the cuts. In the final 10 ml. cut there
was a trace of 2-methoxyoestriol.

7. ATTEMPTED DETECTION OF 16-EPIOESTRIOL IN
   THE MOTHER LIQUORS FROM THE ISOLATION
   OF 2-METHOXYOESTRIOL (Section III, 6)

a. Preliminary

Any 16-epioestriol which might have been
formed from oestriol would have been isolated with
the 2-methoxyoestriol. The mother liquors from
the 2-methoxyoestriol isolation were combined to
give 40 mg. of brownish oil.

b. Acetonide Formation

Due to the small difference in polarity of
2-methoxyoestriol and 16-epioestriol, they cannot
be separated readily by paper or column
chromatography. It was decided to attempt a
separation by acetonide formation. Cis-glycols,
e.g. 16-epioestriol, form an acetonide with
acetone, whereas trans-glycols, e.g. oestriol,
do not. These acetonides can be extracted from
alkali by chloroform, leaving the free phenols in
the alkali. The method used was that of H.J.
Blair and J.B. Brown (personal communication).
The 40 mg. of brown oil were dissolved in 1 ml. of
methanol plus 1 ml. of acetone plus 2 drops of

conc. HCl, and left at room temperature for 1 hr.
The solution was then evaporated to dryness under
vacuum, and the residue dissolved in 20 ml. of
0.1 N-NaOH.   This was extracted three times with
20 ml. portions of chloroform and the chloroform
washed twice with 10 ml. portions of water, the
water washings being added to the alkaline
phase.   The chloroform was dried with sodium
sulphate and evaporated to dryness under vacuum
to yield 5.7 mg. of acetonide fraction.

Carbon dioxide was bubbled through the
alkali till neutral to phenol phthalein and then
extracted three times with 70 ml. portions of
ether.   The ether was washed twice with 20 ml.
portions of water and evaporated to dryness to
give 23.3 mg. of 2-methoxyoestriol fraction.

The acetonide fraction was dissolved in 2 ml.
of methanol and a 500 µg. portion hydrolysed with
10 ml. of 5% (v/v) HCl on a boiling water bath
for half an hour.   This method gives
quantitative hydrolysis of at least 450 µg. of
16-epioestriol acetonide (J.B. Brown, personal
communication).   The hydrolysate was extracted
three times with 30 ml. portions of ether and the
ether washed once with 20 ml. of water.   The

ether was evaporated to dryness to give the hydrolysed acetonide fraction.

Portions (30 µg.) of both the hydrolysed and unhydrolysed acetonide fraction were chromato-graphed in the formamide/chloroform system for 11 hr.   Both fractions ran as single spots identical with 2-methoxyoestriol even though there was a separation of standard 2-methoxy-oestriol from standard 16-epioestriol.

A 30 µg. portion of the hydrolysed acetonide fraction and 20 µg. of authentic 16-epi-oestriol were Kobered in the usual way.   The hydrolysed material behaved in the same way as 2-methoxyoestriol in that a pink colour developed in the first few minutes and then faded in the second stage (Section III, 6, g.). 16-epiOestriol gave a more orange colour which was stable in the second stage of the reaction.

Engel, Baggett and Carter (1957) found that 2-methoxyoestrone was not completely extracted from toluene by N-NaOH, and the same may apply to 2-methoxyoestriol when partitioned between chloroform and 0.1 N-NaOH.   This would account for the presence of 2-methoxyoestriol in the acetonide fraction.

It would thus appear that there is no conversion of oestriol to 16-<u>epi</u>oestriol or to any of the α-ketols under these incubation conditions.

8. <u>CONVERSION OF OESTRIOL TO 16-OXO-OESTRADIOL-17β BY RAT LIVER MICROSOME PLUS SUPERNATANT FRACTION</u>

During experiments on the cofactor requirements for 2-hydroxyoestriol synthesis from oestriol by rat liver microsome plus supernatant fraction, it was found that the addition of nicotinamide to the incubation medium increased the amount of oestriol metabolized without affecting the amount of 2-hydroxyoestriol produced (Section III, 16, d). A trace of a new phenolic metabolite was detected which had the same mobility as 16-oxooestradiol-17β in the formamide/chloroform system. It was ketonic.

On reducing this compound with sodium borohydride it was converted mainly to 16-<u>epi</u>oestriol (as judged from mobility in the formamide/chloroform system) but there was also

a trace of oestriol.   Loke (1958) demonstrated
that a similar reduction of 16-oxooestradiol-17β
brings about a 90% conversion to 16-epioestriol
and 10% to oestriol.

The Fast Black Salt K derivative of this
metabolite had the same mobility as that of
authentic 16-oxooestradiol-17β in the system 30
ethanol:70 water/20 toluene:10 petroleum ether
(40-60°).

It was concluded that this metabolite was
16-oxooestradiol-17β.   No 16α-hydroxyoestrone
could be detected which suggested that the 17β-
hydroxyl group had not been oxidized under these
incubation conditions.

9.   METABOLISM OF OESTRIOL BY RAT KIDNEY
     HOMOGENATES

a. Preliminary Experiments

Investigations on the ability of various rat
tissues to metabolize oestriol (Section III, 16, a)
showed that kidney homogenates could metabolize
appreciable amounts of oestriol but, unlike liver,
did not produce any 2-hydroxyoestriol.

They did, however, produce appreciable amounts of
a compound with the same mobility in the
formamide/chloroform system as 16-oxooestradiol-
17β and a trace of 16-epioestriol.   No other
phenolic metabolites could be detected.

b.   Characterization of these Metabolites

     i.   Girard Separation

     Oestriol (600 µg.) was incubated with 6 ml.
of 0.19 M-potassium phosphate, pH 7.4, plus 6 ml.
of 20% female rat kidney homogenate in 0.25 M-
sucrose plus 3 mg. of DPN^+ plus 3 mg. of TPN^+ for
1 hr. under oxygen at 37°C.   This was then
acidified, extracted with ether as described in
Section III, 12, and subjected to a Girard
separation to produce a ketonic and a non-ketonic
fraction.   This method of carrying out a Girard
separation does not produce any artifactual
16-oxooestradiol-17β from 16β-hydroxy- or 16α-
hydroxyoestrone (Layne and Marrian, 1958).   A
portion of both fractions was chromatographed on
paper in the formamide/chloroform system for 5 hr.
The '16-oxooestradiol' metabolite was ketonic
whilst the '16-epioestriol' compound was non-
ketonic.

The remainder of both fractions was spotted
onto separate sheets of Whatman no. 42 paper and
chromatographed for 5 hr. in the formamide/chloro-
form system.   The areas corresponding to 16-oxo-
oestradiol-17β in the ketonic fraction and to
16-epioestriol in the non-ketonic fraction were
eluted with methanol.

   ii.   16-epiOestriol Fraction

This was further purified by chromatography
on a 1 x 10 cm. celite column using the system
70% methanol/ethylene dichloride.   The 16-epi-
oestriol fraction (2→12 ml.) was collected and
taken to dryness under nitrogen.   This fraction
contained all of the '16-epioestriol' metabolite.

A portion was run on paper in the 70%
acetic acid/ethylene dichloride system for 2½ hr.
and it had the same mobility as 16-epioestriol.

Another portion was heated with Kober
reagent.   It behaved the same as 16-epioestriol
both in the development of the colour and the
absorption curve of the final colour (Marrian
and Bauld, 1955).

The Fast Black Salt K derivative of this
compound had the same mobility as that of

authentic 16-epioestriol in the system 30
ethanol:70 water/20 toluene:10 petroleum ether.

It was concluded that this metabolite was
16-epioestriol.

iii. 16-Oxooestradiol-17β Fraction

On reduction with sodium borohydride this
was reduced mainly to 16-epioestriol plus a
trace of oestriol.   The same result is obtained
with 16-oxooestradiol-17β (Loke, 1958).

The Fast Black Salt K derivative of this
metabolite had the same mobility as that of 16-
oxooestradiol-17β in the system mentioned above.

It behaved the same as 16-oxooestradiol-17β
in the Kober reaction.

The infrared spectrum of this compound was
kindly determined by Dr M.M. Coombs, and was
almost identical with that of 16-oxooestradiol-
17β.   It showed distinct absorption bands at
about 3,600 cm.$^{-1}$(OH) and 1,745 cm.$^{-1}$($>$C = O in
a five-membered ring).   There was a slight
discrepancy in the ratio of intensities of the
3,000 cm.$^{-1}$ band (C-H) : 1,745 cm.$^{-1}$ band.
Dr Coombs suggested that this was due to a

hydrocarbon impurity.

It was concluded that this metabolite was 16-oxooestradiol-17β.

c. Effect of DPN$^+$ and TPN$^+$

The following duplicate incubations were carried out:-

Incubation Tube	Additions
1	100 μg. oestriol in 0.025 ml. PG added just prior to extraction
2	100 μg. oestriol in 0.025 ml. PG added just before incubation
3	100 μg. oestriol in 0.025 ml. PG added just before incubation + ½ mg. DPN$^+$
4	100 μg. oestriol in 0.025 ml. PG added just before incubation + ½ mg. TPN$^+$
5	100 μg. oestriol in 0.025 ml. PG added just before incubation + ½ mg. DPN$^+$ + ½ mg. TPN$^+$

All tubes contained 1 ml. of 0.19 M-potassium phosphate buffer, pH 7.4, plus 1 ml. of 20% female rat kidney homogenate in 0.25 M-sucrose plus 0.98 ml. of water. Incubations were for 1 hr. under oxygen.

After ether extraction (Section III, 12, a), the ether soluble residue was chromatographed on paper in the formamide/chloroform system for 11 hr. Tubes 1 and 2 contained oestriol only.

Tube 3	8 µg. 16-oxo-oestradiol-17β	Trace 16-epi-oestriol
Tube 4	5 µg. 16-oxo-oestradiol-17β	Trace 16-epi-oestriol
Tube 5	15 µg. 16-oxo-oestradiol-17β	Trace 16-epi-oestriol

The amounts given here are as judged by comparison with 10 µg. spots of standard steroid.

No other phenolic metabolites could be detected.

It would thus appear that this oxidation of the 16α-hydroxyl group of oestriol is brought about by a TPN$^+$ or DPN$^+$ requiring dehydrogenase. There does not seem to be a 17β-hydroxy dehydrogenase present in this tissue.

10. METABOLISM OF 16-EPIOESTRIOL AND
17-EPIOESTRIOL BY RAT KIDNEY
HOMOGENATES

a. 16-epiOestriol

Under similar experimental conditions,
16-epioestriol was also converted to 16-oxo-
oestradiol-17β and a trace of oestriol.
Evidence for this was obtained by a Girard
separation of the products followed by Fast
Black Salt K formation, chromatography in the
formamide/chloroform system, and sodium boro-
hydride reduction of the ketonic metabolite.

b. 17-epiOestriol

Incubation of 17-epioestriol under similar
conditions gave two products. These were
subjected to a Girard separation and each product
then chromatographed in the formamide/chloroform
system. They appeared to resemble 16-oxo-
oestradiol-17β and oestriol. However, oxidation
of the 16α-hydroxyl group of 17-epioestriol would
give 16-oxooestradiol-17α. Subsequent enzymic
reduction of the latter compound would produce
mainly 16:17-epioestriol (Breuer, Knuppen and
Pangels, 1959). 16-Oxooestradiol-17α and 16:17-

epioestriol have the same mobility as 16-oxo-
oestradiol-17β and oestriol respectively in the
formamide/chloroform system.

When the 'oestriol' was chromatographed in
the Bush B/S system (100 benzene:55 methanol:45
water) for 25 hr. it migrated 11.7 cm. as
compared with the 8.7 cm. of authentic oestriol.
Breuer, Knuppen and Pangels (1959) have
reported that under similar conditions oestriol
migrated 8.9 cm. and authentic 16:17-epioestriol
11.8 cm.

Sodium borohydride reduction of the '16-
oxooestradiol-17β' showed quite conclusively that
it could not be the 17β compound but that it was
probably the 17α isomer. The main reduction
product was slightly less polar than oestriol in
the formamide/chloroform system but was
appreciably less polar than oestriol when run in
the Bush B/S system for 24 hr. It had the same
mobility as the 'oestriol' metabolite which
suggested that it was in fact 16:17-epioestriol.
The minor reduction product had the same
mobility as 17-epioestriol in the formamide/
chloroform system. No 16-epioestriol could be
detected.

As estimated from the Folin and Ciocalteu colour
the yields of these metabolites are given in the
Table below.   Incubation conditions were as
described above in section 9, c, tube 5.

Steroid Incubated	Metabolites
Oestriol	15-20% 16-oxooestradiol-17β; 1% 16-epioestriol
16-epiOestriol	5% 16-oxooestradiol-17β; 1% oestriol
17-epiOestriol	15% 16-oxooestradiol-17α; 1% 16:17-epioestriol

16:17-epiOestriol was not tested, but presumably
would give 16-oxooestradiol-17α and 17-epioestriol.

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11. DISCUSSION

This work shows quite conclusively that
16α-hydroxyoestrone can be converted to oestriol
by rat liver slices.   Breuer, Nocke and Knuppen
(1958, 1959a) have confirmed this with rat,
guinea-pig and human liver slices.   One major
difference in the experimental procedure of
Breuer's group is that all of their incubations
were carried out under air.   As shown in part
4, c. of this Section this can apparently result
in lower yields of steroid.   This effect is
discussed at the end of Section III.   Thus
with rat liver slices, these workers obtained a
6% conversion of 16α-hydroxyoestrone to oestriol
as compared with the 18% described in this thesis.
Similarly, they could detect no 17-epioestriol
whereas a trace of this compound was found with
the methods adopted in the present experiments.
When human liver slices were used they did
detect a trace of 17-epioestriol.   Brown and
Marrian (1957) have shown that after injection
of 16α-hydroxyoestrone into a male and a post-
menopausal woman, over 30% was excreted in the
urine as oestriol in both cases.   There was no
detectable increase in the amount of oestrone or

oestradiol-17β excreted.

Oestradiol-17β can be converted in small yield into 16α-hydroxy- and 16β-hydroxyoestrone and 16-oxooestradiol-17β by rat liver slices (Breuer, Nocke and Knuppen, 1959b). Thus, rat liver possesses all the enzymes necessary for the conversion of oestrone to oestriol via 16α-hydroxyoestrone.

16β-Hydroxyoestrone has been isolated from pregnancy urine (Layne and Marrian, 1959) and as a urinary metabolite of $\left[16^{14}C\right]$ oestradiol-17β in man (Brown, Fishman and Gallagher, 1958). Whether 16α- and 16β-hydroxylation is accomplished by the same or different enzymes is not known. The experiments of Breuer and Nocke (1959) indicate that human liver slices metabolize 16β-hydroxyoestrone mainly to 16-epi-oestriol plus a trace of 16:17-epioestriol. These authors also made the very interesting observation that if 16β-hydroxyoestrone is allowed to stand in Krebs' phosphate saline, pH 7.4, for 1 hr. at 37°C, there is a 40% conversion to the more stable isomer, 16-oxo-oestradiol-17β. Thus, 16β-hydroxyoestrone can undergo the following transformations:-

16β-Hydroxy-
oestrone

16-epiOestriol

16-Oxo-
oestradiol-17β
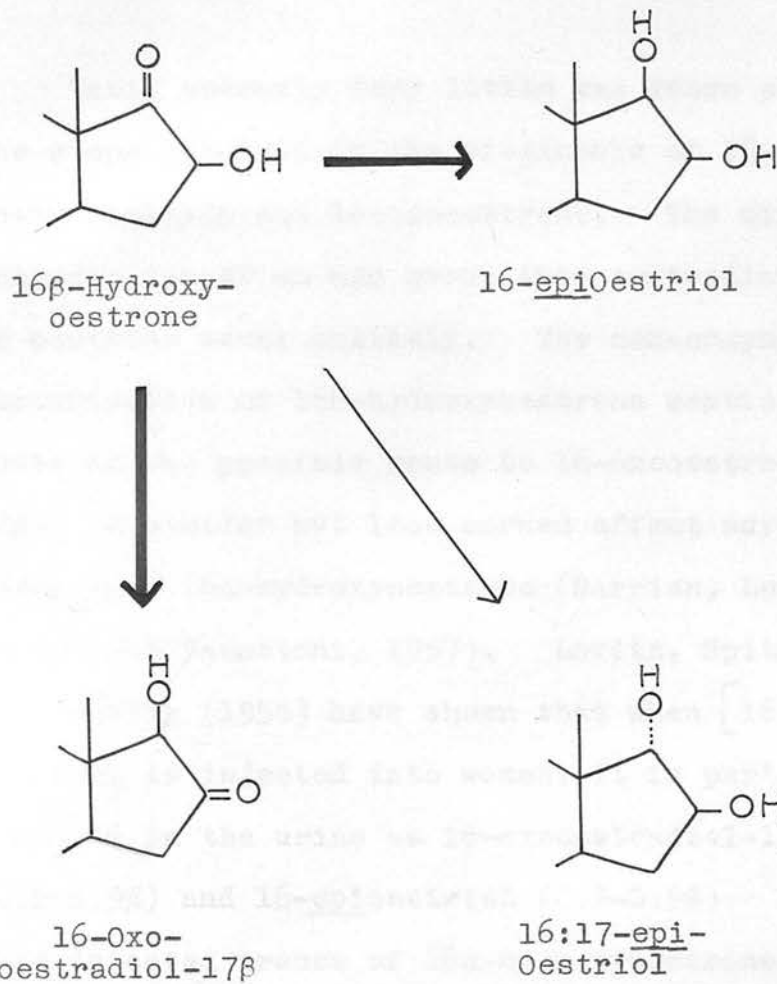
16:17-epi-
Oestriol

16-epiOestriol has been isolated from pregnancy
urine by Marrian and Bauld (1955). 16:17-epi-
Oestriol has not yet been isolated from this
source.

Until recently very little was known about
the steps involved in the biogenesis of 16-oxo-
oestradiol-17β and 16-oxooestrone. The direct
introduction of an oxo group into oestradiol-17β
or oestrone seems unlikely. The non-enzymic
isomerization of 16β-hydroxyoestrone mentioned
above is one possible route to 16-oxooestradiol-
17β. A similar but less marked effect may
occur with 16α-hydroxyoestrone (Marrian, Loke,
Watson and Panattoni, 1957). Levitz, Spitzer
and Twombly (1958) have shown that when $\left[16^{14}C\right]$-
oestriol is injected into women, it is partly
excreted in the urine as 16-oxooestradiol-17β
(0.2-0.9%) and 16-epioestriol (0.3-0.5%). They
also detected traces of 16α-hydroxyoestrone and
16-oxooestrone, but the amounts were very small
and radiochemical purity was not established.
This indicated that 16-oxooestradiol-17β could
be formed by oxidation of oestriol. This has
been confirmed by the in vitro experiments with
rat kidney homogenates described in this Section.
The 16-hydroxyl group of 16-epioestriol and 17-epi-
oestriol can also be oxidized by this tissue
preparation. Rat liver slices, homogenates and

cell fractions did not appear to possess this 16-
hydroxy dehydrogenase activity, but when
nicotinamide was added to a rat liver microsome
plus supernatant fraction supplemented with DPN^+
plus TPN^+, a small dehydrogenase activity was
shown.   No 17-hydroxy dehydrogenase activity
could be demonstrated with either of these
organs.   Breuer, Knuppen and Pangels (1959)
claim that rabbit liver possesses a weak 16-
hydroxy and 17-hydroxy dehydrogenase activity.

16-Oxooestradiol-17β can thus be formed in
the following ways:-

16β-Hydroxy-
oestrone

16α-Hydroxy-
oestrone

16-Oxo-
oestradiol-17β

Oestriol

16-epiOestriol
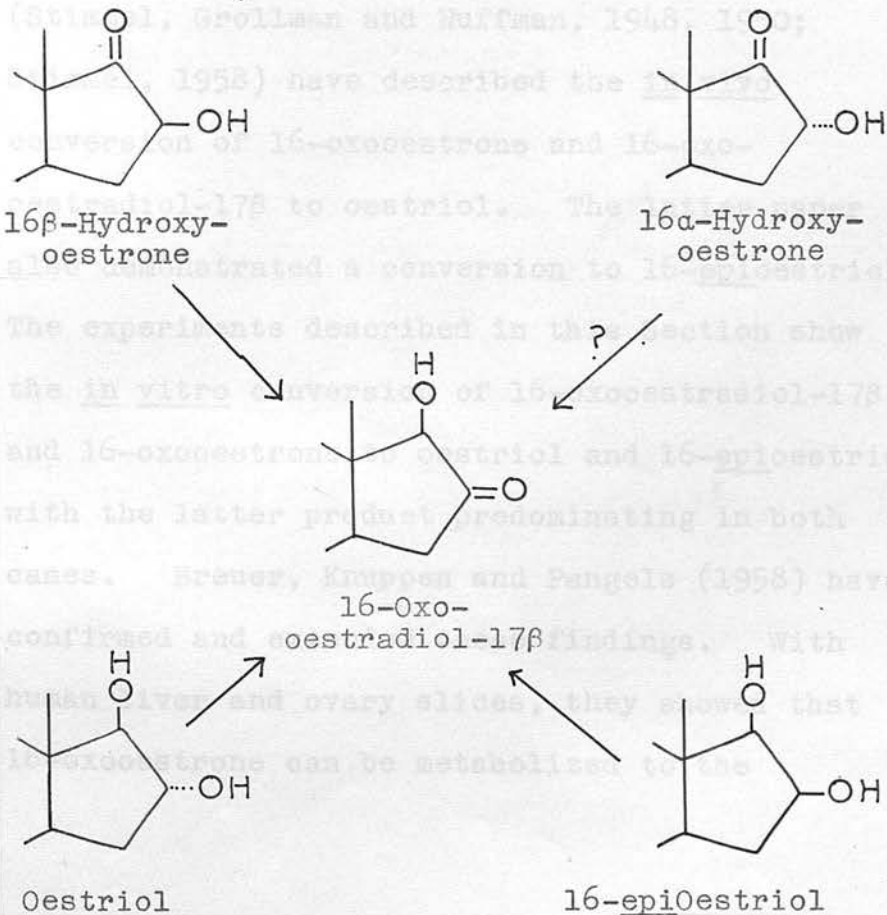
Because of the large amounts of oestriol produced
by humans, the main route is probably by oxidation
of oestriol in the kidney.   Experiments with
human kidney will have to be carried out before
any definite conclusions can be reached on this
point.

16-Oxooestrone could be formed by oxidation
of 16-oxooestradiol-17β, but a more likely path-
way is from 16α-hydroxy- and 16β-hydroxyoestrone
in the kidney.   The kidney 16-hydroxy
dehydrogenase has not yet been tested with either
of these compounds.   A number of papers
(Stimmel, Grollman and Huffman, 1948, 1950;
Stimmel, 1958) have described the in vivo
conversion of 16-oxooestrone and 16-oxo-
oestradiol-17β to oestriol.   The latter paper
also demonstrated a conversion to 16-epioestriol.
The experiments described in this Section show
the in vitro conversion of 16-oxooestradiol-17β
and 16-oxooestrone to oestriol and 16-epioestriol
with the latter product predominating in both
cases.   Breuer, Knuppen and Pangels (1958) have
confirmed and extended these findings.   With
human liver and ovary slices, they showed that
16-oxooestrone can be metabolized to the

following compounds:-

16β-Hydroxy-
oestrone

16-<u>epi</u>Oestriol

16-Oxo-
oestrone

16-Oxo-
oestradiol-17β

Oestriol

16α-Hydroxy-
oestrone

17-<u>epi</u>Oestriol
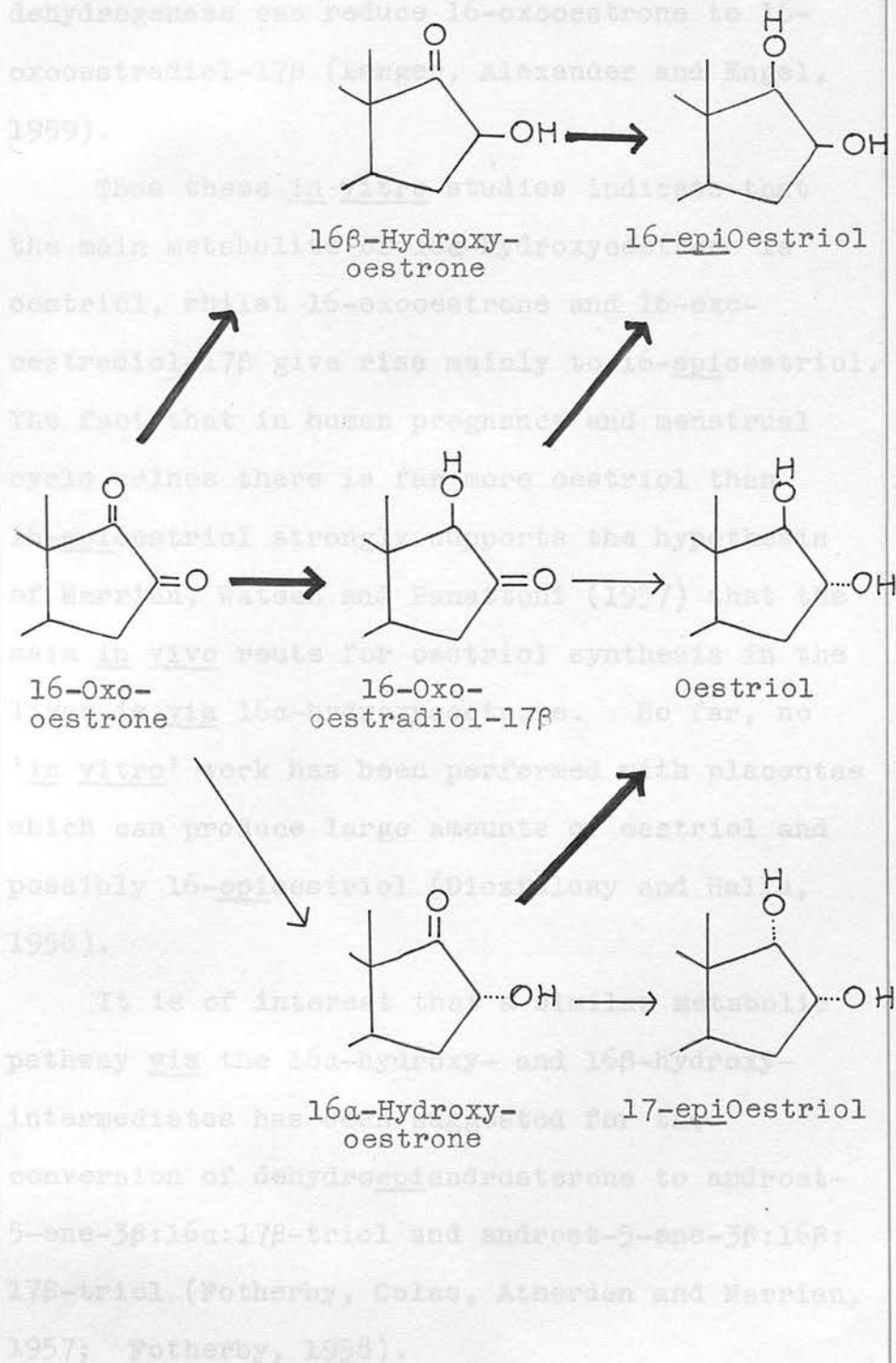
Purified human placental oestradiol-17β
dehydrogenase can reduce 16-oxooestrone to 16-
oxooestradiol-17β (Langer, Alexander and Engel,
1959).

Thus these in vitro studies indicate that
the main metabolite of 16α-hydroxyoestrone is
oestriol, whilst 16-oxooestrone and 16-oxo-
oestradiol-17β give rise mainly to 16-epioestriol.
The fact that in human pregnancy and menstrual
cycle urines there is far more oestriol than
16-epioestriol strongly supports the hypothesis
of Marrian, Watson and Panattoni (1957) that the
main in vivo route for oestriol synthesis in the
liver is via 16α-hydroxyoestrone. So far, no
'in vitro' work has been performed with placentae
which can produce large amounts of oestriol and
possibly 16-epioestriol (Diczfalusy and Halla,
1958).

It is of interest that a similar metabolic
pathway via the 16α-hydroxy- and 16β-hydroxy-
intermediates has been suggested for the
conversion of dehydroepiandrosterone to androst-
5-ene-3β:16α:17β-triol and androst-5-ene-3β:16β:
17β-triol (Fotherby, Colas, Atherden and Marrian,
1957; Fotherby, 1958).

Although the _in vivo_ conversion of oestrone
and oestradiol-17β to oestriol has been known for
some time, the _in vitro_ conversion has been
demonstrated only recently. Hagopian (1955)
obtained a 1-1.5% conversion of $\left[16^{14}C\right]$-
oestradiol-17β to oestriol with rat liver slices,
and Engel, Baggett and Halla (1958) obtained a
3.8% conversion with human foetal liver slices.
The facility with which 16α-hydroxyoestrone is
converted to oestriol by liver slices suggests
that the low conversion of oestradiol-17β to
oestriol is due to the low activity of the 16-
hydroxylating enzyme and that this is the rate
limiting step in the overall conversion.
Another possibility which has not been fully
investigated is that the 16-hydroxylation occurs
outside the liver. Loke (1958) has put forward
evidence that the adrenal cortex can 16α-
hydroxylate oestrone, but the activity was very
low.

Fig. 4 is a representation of the possible
in vitro conversions of oestrone and its
metabolites.

Some interesting stereochemical considerations
arise from these results. Fig. 5 is a

FIG. 4.   IN VITRO INTERCONVERSIONS OF OESTRONE AND ITS METABOLITES.
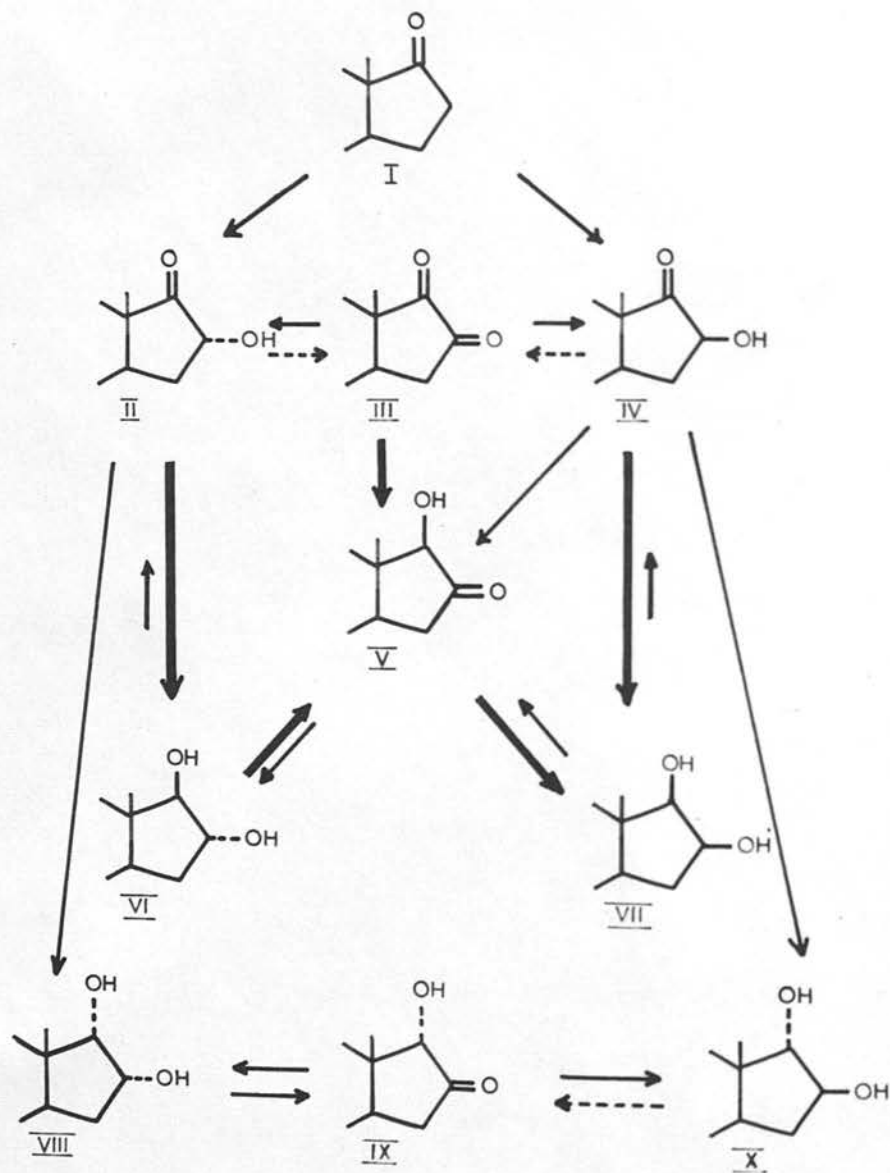
I   OESTRONE.

II   16α-OH OESTRONE.

III 16-OXO OESTRONE

IV   16β-OH OESTRONE

V   16-OXO OESTRADIOL - 17β.

——→ MAJOR PATHWAY.

VI   OESTRIOL.

VII 16-EPIOESTRIOL.

VIII 17-EPIOESTRIOL.

IX   16-OXO OESTRADIOL - 17α.

X   16,17-EPIOESTRIOL.

----→ HYPOTHETICAL PATHWAY.

FIG.5.   3-DIMENSIONAL REPRESENTATION OF THE C & D RINGS
OF 16ɗ-OH OESTRONE AND 16β-OH OESTRONE.

————— β BOND.   - - - ɗ BOND.
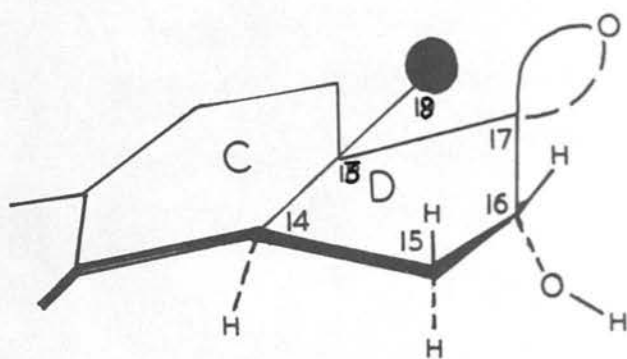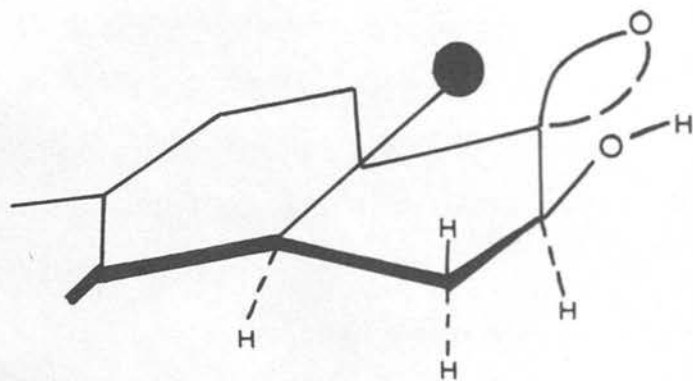
16ɗ-OH OESTRONE.

16β-OH OESTRONE.

diagrammatic three-dimensional representation of 16α-hydroxy- and 16β-hydroxyoestrone. A broken line represents an 'α' bond, i.e. towards the 'back' of the molecule, and a solid line a 'β' bond, i.e. towards the 'front' of the molecule.

The large 18β angular methyl group effectively blocks the front of the 17-oxo group, thus making enzymic attack from the front of the molecule difficult at this position. It would, therefore, be expected that enzymic reduction of a 17-oxo group would produce mainly the 17β isomer, i.e. the 'α' bond of the oxo group would be broken. Thus oestrone would be expected to give mainly oestradiol-17β, 16α-hydroxyoestrone oestriol, and 16β-hydroxyoestrone 16-epioestriol. This is in fact what has been observed experimentally. The fact that small amounts of the 17α isomers are formed suggests that this steric hindrance is not complete. However, some species, e.g. rabbit (Heard, Bauld and Hoffman, 1941), can produce appreciable amounts of oestradiol-17α from oestrone. Substrate specificity studies on purified human placental oestradiol-17β dehydrogenase agree with the above

considerations in that oestradiol-17α is not
attacked by this enzyme (Langer, Alexander and
Engel, 1959).   These authors also showed that an
`α´ angular methyl group or a 16α-hydroxy group
prevented oxidation of the 17β-hydroxy group,
whereas the presence of a 16β-hydroxy did not
produce complete inhibition.

That the steric hindrance produced by the
16-hydroxyl group is not as important as the
angular methyl group is suggested by the fact
that 16α-hydroxyoestrone produces mainly oestriol
with only traces of the 17α isomer.

The importance of the 'α' side of the
molecule is also suggested by the action of the
kidney 16-hydroxy dehydrogenase.   This can only
oxidize the 16β-hydroxy group of 16-epioestriol
to about one-third of the extent of the 16α-
hydroxy group of oestriol and 17-epioestriol.
As only a crude kidney homogenate was used in
these experiments, it will need further
purification before any definite conclusions can
be drawn about this enzyme.

1. INTRODUCTION

In 1934 Zondek, using a bioassay technique demonstrated that rat and guinea-pig liver slices would deactivate oestrone and that this was an oxidative process. He also showed that acid hydrolysis of the incubation medium did not increase the bioactivity of the medium. This ruled out conjugation as a pathway of any significance. This work has been confirmed by a number of workers using bioassay techniques

SECTION III

OXIDATIVE METABOLISM OF OESTRIOL

(Dingemanse and Laqueur, 1937).

The oxidative metabolism of oestrogens by liver preparations of a number of species has also been demonstrated by numerous workers using more refined chemical and physical estimation and isolation methods. Thus Ryan and Engel (1953), using countercurrent distribution and fluorimetric analysis, showed that the conversion of oestrone to oestradiol-17β by rat liver slices was increased if the incubations were carried out anaerobically. The total recovery of added oestrogen was also enhanced. Similarly, Heigel and Mueller (195.) have demonstrated that the irreversible protein binding of $[6,7-{}^{14}C]$ oestradiol-17β by fortified rat liver homogenates is 93%

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inhibited if the incubation is carried out under nitrogen.

A number of oxidized oestrogens have been isolated in the past few years from various sources. 6α-Hydroxyoestradiol-17β and 6-oxo-oestradiol-17β have been isolated as liver microsomal metabolites of $[16^{14}C]$oestradiol-17β (Mueller and Rumney, 1957). Loke (1958) has shown that oestrone can be hydroxylated in the 18 position by ox adrenals and 18-hydroxyoestrone has been isolated from pregnancy urine (Loke, Marrian, Johnson, Meyer and Cameron, 1958). In 1957 two independent groups of workers demonstrated that 2-methoxyoestrone was a urinary metabolite of $[16^{14}C]$oestradiol-17β in humans (Kraychy and Gallagher, 1957; Engel, Baggett and Carter, 1957). This was followed by the isolation of this compound from pregnancy urine (Loke and Marrian, 1958). 2-Methoxylation of the A ring seems to be a fairly general metabolic pathway. Thus 2-methoxy derivatives of oestriol (Fishman and Gallagher, 1958), oestradiol-17β (Frandsen, 1959), oestradiol-17α (Stimmel, 1959) and possibly of 16α-hydroxyoestrone and 16-oxo-oestradiol-17β (Loke, 1958) have been isolated

from urine under various conditions.

By analogy with the formation of 3 O-methyl adrenalin (Axelrod and Tomchick, 1958) and other O-methyl compounds (Pellerin and D'Ionio, 1958), it would seem likely that the introduction of a methoxy group into the steroid nucleus is a two-stage process. The first step would be a hydroxylation ortho to the existing phenolic group, followed by methylation. If this is the correct pathway then a number of 2-hydroxylated oestrogens should also exist. Garst and Friedgood (1952) claim to have isolated an o-hydroxylated oestriol from urine, but their evidence is far from convincing.

Hecker and Mueller (1958), using a model compound, $[8^{14}C]$ tetrahydro-2-naphthol, have shown that rat liver microsomes can convert this compound into a p-quinol.

They also claim to have isolated the corresponding quinol of oestradiol-17β (Mueller, Herranen and Jervel, 1958).

In addition to the hydroxylations quoted above, Levitz, Spitzer and Twombly (1958) have shown that $\left[16^{14}C\right]$ oestriol, when injected into women is partly excreted as 16-oxooestradiol-17β and partly as 16-epioestriol. There may also have been a trace of 16α-hydroxyoestrone. This would suggest that in some cases the ring D hydroxyl groups can be oxidized to the corresponding oxo group.

During experiments on the conversion of 16α-hydroxyoestrone to oestriol by rat liver slices (Section II) it was found that the yield of oestriol was increased if the incubations were carried out under nitrogen. Following up this observation, it was found that when 100 μ g. of oestriol were incubated with rat liver slices under oxygen, only about 12 μ g. could be recovered. In view of the magnitude of this oxygen effect and the number of possible metabolites, it was decided to investigate further the oxidative metabolism of oestriol.

2. EXPERIMENTAL METHODS

Most of the methods used in these
experiments are described in Section I, 2.

a. Preparation of Homogenates

Liver and kidney homogenates were prepared
as described in Section I, 2. In the
preparation of the ovarian and uterine
homogenates the initial mincing was omitted.
The ovaries were homogenized in an 0.8 x 15 cm.
glass tube with a hand-operated metal plunger.

The uteri were slit longitudinally and the
inside surface blotted dry with filter paper.
They were then cut into small pieces and then
finely minced with a Mickle automatic slicer.
This brei was added to the appropriate volume of
0.25 M-sucrose to give a 20% (w/v) suspension
which was not further treated.

b. Absorption Spectra

All coloured solutions were read in a
Unicam S.P.600. All ultraviolet absorption
spectra were measured in a Unicam S.P.500.

3. PRELIMINARY EXPERIMENTS

The following preliminary incubation was carried out:-

Tubes 1, 2, 5 and 6: 100μg. of oestriol in
0.05 ml. of PG + 2 ml. of
oxygenated Krebs' phosphate
saline, pH 7.4, + 200 mg.
of male rat liver slices.

Tubes 3 and 4:   The same as 1, 2, 5 and 6
except that the saline was
treated with nitrogen
instead of oxygen.

Tubes 1, 2, 5 and 6 were incubated for 2 hr. at 37°C under oxygen.   Tubes 3 and 4 were incubated under nitrogen for the same period.

In view of the alkaline lability of some of the newly isolated oestrogens (Layne and Marrian, 1958), it was decided to omit the phenol separation from the extraction.   The extraction procedure used was as follows:-

1.   The medium was homogenized in the reaction vessel.

2.   15 ml. of 15% (v/v) HCl were added.

3.   The medium was extracted three times with 50 ml. of ether and the ether phase evaporated to dryness under vacuum on a hot water bath.

4. The brown oily residue was leached with
5 ml. of 70% aqueous methanol and the methanol
washed twice with 5 ml. of n-hexane. The hexane
was discarded. This hexane wash removes most of
the very non-polar lipids.

5. The methanol phase was taken to dryness
under vacuum and the residues of tubes 1, 2, 3
and 4 chromatographed on paper in the formamide/
chloroform system for 15 hr. The oestriol spots
were much more intense in tubes 3 and 4 than in
1 and 2. All four tubes contained a less polar
metabolite which ran between 16-epi- and 17-epi-
oestriol in this system but the amount of this
material in tubes 1 and 2 (about 20μ g.) was
much greater than in tubes 3 and 4. This
metabolite was called OMI (oestriol metabolite I).
There was also a Folin and Ciocalteu positive
area at the origin in tubes 1 and 2. This
behaved anomalously in that a blue colour
developed before the paper was exposed to
ammonia. The blue colour intensified in the
presence of ammonia. This was called OMII.

The residues of tubes 5 and 6 were combined
and subjected to a Girard separation. The
ketonic and non-ketonic fractions were
chromatographed in the formamide/chloroform

system for 15 hr.   Both OMI and OMII were non-ketonic.

One of the major metabolic modifications undergone by steroids is hydroxylation. This occurs in a number of positions depending on the steroid involved and the tissue used.   It is characteristic of these hydroxylations that the oxygen of the hydroxyl group is derived from molecular oxygen and not from water (Hayano and Dorfman, 1955).   Thus the oxygen requirement for oestriol metabolism suggested the introduction of one or more hydroxyl groups into the molecule.   This would account for the increased polarity of OMII, but would not fit in with the decreased polarity of OMI.

About this time, Loke (1958) had isolated two compounds from the phenolic ketonic fraction of pregnancy urine which he thought were 2-methoxy-16α-hydroxyoestrone (compound A) and 2-methoxy-16-oxooestradiol-17β (compound B).   On reduction of compound A with sodium borohydride he obtained a compound which on his hypothesis was 2-methoxyoestriol.   This reduction product of compound A had the same mobility in the formamide/chloroform system as OMI.

If OMI was 2-methoxyoestriol, then OMII might be the corresponding 2-hydroxy compound. The only way to identify OMI and OMII with certainty was by their large scale isolation.

4. LARGE SCALE ISOLATION OF OMI AND OMII

Incubations were carried out in stoppered 250 ml. conical flasks. Each flask contained 6 mg. of oestriol in 2.5 ml. of PG plus 50 ml. of Krebs' phosphate saline (oxygenated), pH 7.4, plus 5 g. of rat liver slices. Incubations were for 4 hr. at 37°C under oxygen. The flasks were reoxygenated after 2 hr. At the end of the incubation the contents of the flasks were combined and homogenized in a Waring Blendor. For each fifteen flasks the following extraction procedure was used:-

1. 150 ml. of 15% (v/v) HCl were added and extracted four times with 500 ml. of ether.

2. The ether was evaporated to dryness under vacuum and the oily residue extracted four times with 15 ml. of 70% aqueous methanol.

3. The methanolic extract was washed twice with 15 ml. of n-hexane and the methanol taken to dryness under vacuum.

4. The residue was transferred to a number of Whatman no. 42 papers and chromatographed in the formamide/chloroform system for 12 hr. A small strip from each paper was sprayed with Folin and Ciocalteu reagent to locate the phenols. A complete separation of oestriol from OMI was obtained, but the separation of oestriol and OMII was by no means complete. Accordingly, the OMII eluted from these chromatograms contained an appreciable amount of oestriol and was called the 'oestriol fraction'.

The mobile phase which had run off the bottom of the papers was taken to dryness and worked up later as the 'eluate fraction' (Section II, 6).

The 'oestriol fraction' was rechromatographed in the same system for 48 hr. The OMII had still not moved from the origin and had not been completely separated from the oestriol. The OMII was extracted with methanol as before and rechromatographed in the same system for 60 hr. The OMII had just started to migrate and was completely separated from the oestriol. The OMII was eluted with methanol.

From 456 mg. of oestriol, 21 mg. of OMI and 13.5 mg. of OMII were obtained.

5. PRELIMINARY IDENTIFICATION OF OMI

a. Purification of the Crude OMI

The 21 mg. of OMI were dissolved with warming in 0.1 ml. of 1 acetone:1 benzene. On standing overnight at -17°C there was a slight precipitation. This was not satisfactory and the mixture was taken to dryness. It was dissolved in a small volume of warm acetone and an equal volume of benzene was added. This mixture was gently warmed and as the volume decreased benzene was added to keep the volume constant. When the solution turned cloudy it was left to stand at room temperature. At this stage the solvent was probably pure benzene. White gelatinous 'crystals' came down which were washed with benzene and dried in vacuo to give 10.4 mg. of whitish solid.

b. Carbon and Hydrogen Analysis

This material was dried at 100°C for 1 hr. without loss of weight and contained 70.48% C and 7.80% H. There was no residue. This corresponds to the analysis of 2-methoxyoestriol ($C_{19}H_{26}O_4$) containing 1 molecule of acetone of crystallization (C_3H_6O). This was not satisfactory.

c.   Ultraviolet Absorption Spectrum

A sample of OMI (118μ g.) was dissolved in 3 ml. of ethanol and the u.v. spectrum measured. Assuming a molecular weight of 374 ($C_{22}H_{30}O_5$), the following results were obtained:- $\lambda_{max.}$ 288 mμ $\epsilon = 4600$ $\lambda_{min.}$ 253 mμ $\epsilon = 634$. Fishman and Gallagher (1959) have reported the following values for 2-methoxyoestriol:- $\lambda_{max.}^{ethanol}$ 286 mμ $\epsilon = 3700$ $\lambda_{min.}^{ethanol}$ 253 mμ $\epsilon = 350$.

This provided further evidence that OMI was 2-methoxyoestriol as, apart from 6-oxygenated derivatives, most oestrogens have a $\lambda_{max.}$ 280 m μ. By comparing the extinction coefficients of this material with those for authentic 2-methoxyoestriol, this batch of OMI was 85-90% pure.

When 0.25 ml. of N-NaOH was added to the ethanolic solution of OMI, the 288 mμ peak shifted to 300 mμ ($\epsilon = 5266$) and a new maximum appeared at 245 mμ ($\epsilon = 7990$).   The corresponding minima were at 233 mμ ($\epsilon = 5344$) and 273 mμ ($\epsilon = 1432$).   2-Methoxyoestrone develops a maximum at 245 mμ under similar conditions.

Whilst the above evidence suggested that OMI
was 2-methoxyoestriol, it was by no means
conclusive, so a fresh batch of OMI was prepared
as before (Section III, 4).

6. ISOLATION OF CRYSTALLINE OMI AND ITS
IDENTIFICATION AS 2-METHOXYOESTRIOL

From 550 mg. of oestriol, 96 mg. of OMI and
33 mg. of OMII were obtained.

a. Charcoal Treatment

As the first batch of OMI was impure, a
different purification method was tried with
the second batch. The 96 mg. were dissolved in
ethanol, warmed with a small amount of Norit
charcoal and filtered whilst still warm. The
filtrate was taken to dryness, redissolved in
ethanol and refiltered in order to remove all
traces of charcoal. This removed some of the
contaminating pigment.

b. Alumina Chromatography

In the trial experiments, crude OMI from the
mother liquors of the first batch was used.
An alumina column, 0.7 x 6 cm., was prepared
in benzene. Savory and Moore alumina
deactivated with 5% of water was used. About

1 mg. of OMI in benzene was put onto the column
and eluted using a discontinuous gradient of
methanol in benzene.   Each 10 ml. cut was
chromatographed in the formamide/chloroform
system for 15 hr. to detect any OMI.   Most of
the OMI was eluted by 40-70 ml. of 1% methanol
in benzene.   When the solvent was changed to 3%
methanol in benzene the remainder was eluted.
This looked hopeful as most of the pigment
remained at the top of the column.   However, the
fact that the OMI was eluted with 1% methanol in
benzene meant that any less polar contaminating
material might also be eluted in the same
fraction.   The experiment was repeated using
alumina deactivated with 2% of water.   No OMI
was eluted by 70 ml. of 0.5%, 50 ml. of 1%, or
40 ml. of 1.5% methanol in benzene.   All of the
OMI was eluted by 150 ml. of 3% methanol in
benzene.   As all the pigment remained at the top
of the column, this method was used to purify
the OMI.

A column, 1.5 x 5 cm., was prepared and 20 mg.
of OMI in benzene were put on to the column.
No OMI was eluted by 50 ml. each of 0.5%, 1% and
1.5% methanol in benzene.   All the OMI was

eluted by 20→250 ml. of 3% methanol in benzene.

The remainder of the OMI (42 mg.) was treated in

a similar way on another column.   This resulted

in considerable purification of the OMI.

c.   Crystallization and Melting Point Determination

The OMI (54 mg.) was leached twice with 0.1

ml. of acetone.   This removed most of the

remaining pigment leaving 27 mg. of whitish solid.

This material was crystallized from methanol-

benzene at room temperature.   The crystals were

washed twice with 0.2 ml. of benzene and

recrystallized from methanol-benzene.   The

crystalline product, weighing 21.8 mg., appeared

white, but on microscopic examination was seen to

be contaminated with oily material.   It melted

on a hot stage apparatus at 215-219°C with

decomposition.   This material was leached twice

with 0.1 ml. of benzene and crystallized from

methanol-benzene.   This gave 15.4 mg. of white

crystals and had m.p. 213-215°C, with softening

at 212°C (evacuated sealed tube).   2-Methoxy-

oestriol has m.p. 215-218°C (Fishman and

Gallagher, 1959).

d.   Optical Rotation

OMI (2.539 mg.) was dissolved in 0.5 ml. of

FIG. 6.   KOBER ABSORPTION CURVE OF 2-METHOXY OESTRIOL.

20 μg. OESTRIOL.

54 μg. 2-METHOXY OESTRIOL.

15 μg. 2-METHOXY OESTRIOL HEATED FOR
4 Mins. WITH KOBER REAGENT.

OPTICAL DENSITY.

WAVELENGTH mμ.
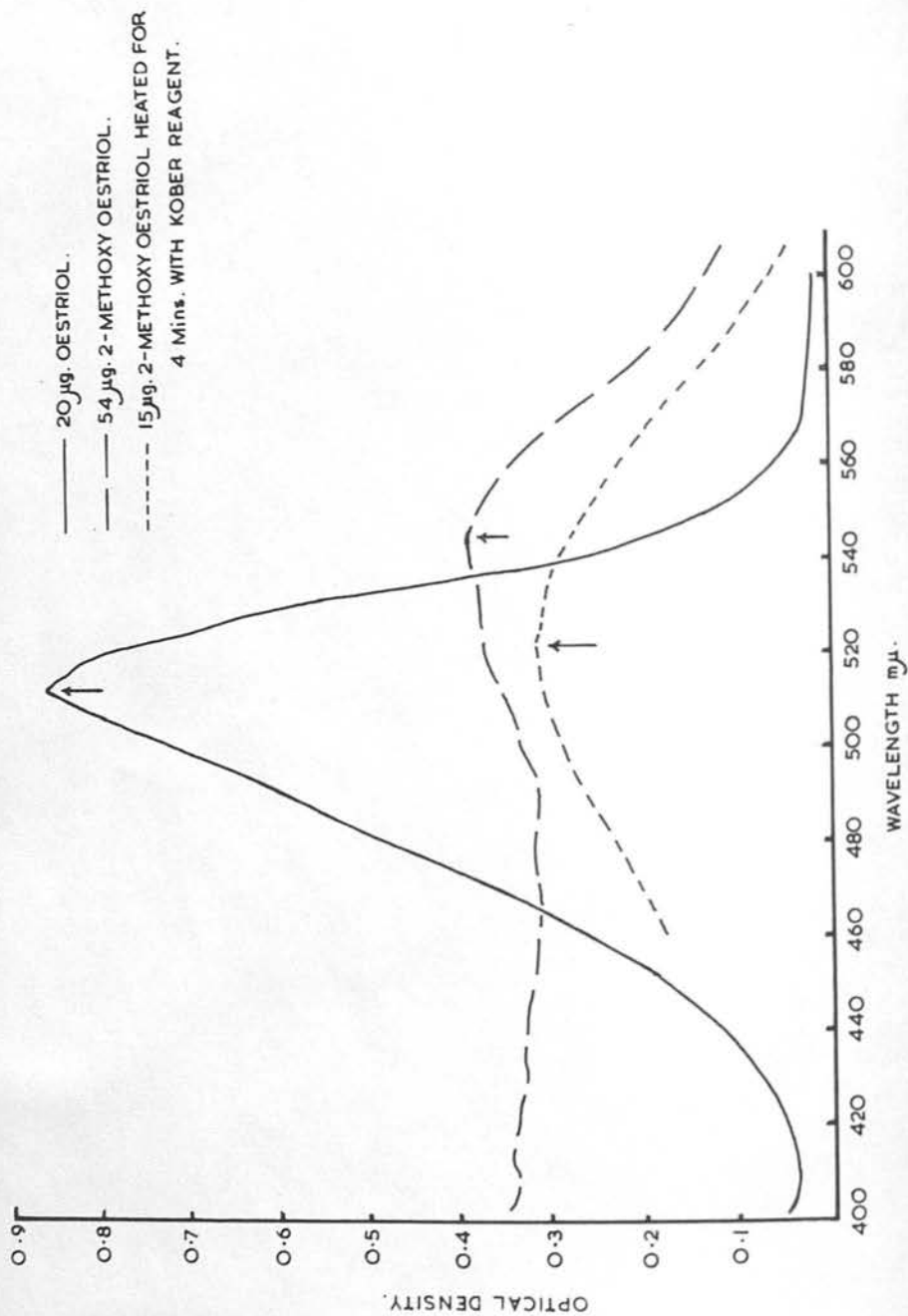
ethanol and the optical rotation measured -

$[\alpha]_D^{17.2}$ = +81.1°. 2-Methoxyoestriol -

$[\alpha]_D^{25}$ = +83° (ethanol) (Fishman and Gallagher, 1959).

e. Infrared Spectrum

Dr Gallagher kindly measured the i.r. spectrum of OMI in chloroform and reported that it was identical with authentic 2-methoxyoestriol in every respect.

f. Ultraviolet Absorption Spectrum

OMI (109 μ g.) was dissolved in 3 ml. of ethanol and its u.v. spectrum measured. $\lambda_{max.}$ 287 mμ (ϵ = 3980) $\lambda_{min.}$ 253 mμ (ϵ = 676). Both of these extinction coefficients are high as compared with the figures quoted by Fishman and Gallagher (1959) $\lambda_{max.}^{ethanol}$ 286 mμ (ϵ = 3700) $\lambda_{min.}$ 253 mμ (ϵ = 350).

g. Kober Reaction

OMI (54 μ g.) was Kobered with oestriol reagent. A reddish-pink colour developed after 2 min. heating in the first stage. This colour reached maximum intensity after about 5 min. and then faded. In the second stage the colour faded slightly. The final colour had a flat absorption peak at 540-547 mμ. This is similar

to 2-methoxyoestrone (Loke, 1958) (Fig. 6).

It was concluded that OMI was 2-methoxyoestriol.

7. PRELIMINARY IDENTIFICATION OF RAT OMII

a. Purification of Crude OMII

The 21 mg. of OMII from the first batch were leached with 0.3 ml. of ether and then 0.3 ml. of ethyl acetate without removing any appreciable amounts of pigment. On leaching with a small volume of acetone, some pigment was removed, leaving 10.7 mg. of brownish gum. This was dissolved in a small volume of methanol and treated with Norit charcoal (Section III, 6, a). There was only a slight decolourization of the OMII. The dry residue was dissolved in 0.5 ml. of warm acetone and water added dropwise. When 0.3 ml. of water had been added the solution turned cloudy, and on standing at -17°C overnight produced 8 mg. of whitish crystals.

b. Melting Point Determination

In an evacuated sealed tube it began to sublime about 220°C, softened at 250°C, and slowly merged into a melt at about 260°C. The real melt was probably in the region of 262-263°C.

There was no apparent decomposition. Obviously the OMII was still impure.

c. Ultraviolet Absorption Spectrum

Its u.v. spectrum in ethanol showed maximum absorption at 287-289 mν with a minimum at 254 mν. This suggested that it might be related to 2-methoxyoestriol.

8. STABILITY OF OMII TO A NUMBER OF EXPERIMENTAL PROCEDURES

In these experiments the OMII, after the treatment described, was chromatographed on paper in the 70% acetic acid/ethylene dichloride system to detect the OMII.

a. Repeated Evaporation of a Methanolic Solution of OMII in Air and Nitrogen

The OMII was dissolved in 0.25 ml. of methanol and evaporated under a gentle stream of gas in a boiling water bath. This was repeated five times. In the nitrogen tube there was very little, if any, loss of OMII. In the air tube most of the OMII had been destroyed. Chromatography, however, showed a very pronounced spot at the origin in the air tube.

b. Treatment with Alkali and Acid

See also Section III, 10, e.

OMII (200μg.) was completely destroyed on standing in 0.1 N-NaOH for 1 hr. at room temperature in air. On adding the alkali to the dry OMII, an immediate orange colour developed.

On the other hand, OMII appeared to be quite stable in glacial acetic acid. Dr M.M. Coombs (personal communication) has shown that 2-hydroxy-oestriol is stable below pH 9, but is rapidly decomposed in more alkaline solutions. The nature of this alkaline degradation is discussed in Section III, 10, e.

c. Alumina Chromatography

An alumina column (0.7 x 6 cm.) was prepared as described in Section III, 12, b. An aliquot of OMII in benzene was transferred to the column and eluted using a discontinuous gradient of increasing amounts of methanol in benzene. Fractions of eluate (20 ml.) of each solvent mixture were collected. No OMII was eluted by 1%, 3%, 4%, 5%, 7% or 10% of methanol in benzene. Under these conditions oestriol is eluted by 4% of methanol in benzene.

d. Partition Chromatography

A 1 x 10 cm. celite column was prepared for use with the system 70% methanol/ethylene dichloride (Section I, 2, h). The OMII was eluted by 25→30 ml. of ethylene dichloride, but recovery was by no means quantitative. Under similar conditions, oestriol is eluted by 10→30 ml. of ethylene dichloride.

Obviously OMII is not very stable, especially under oxidative conditions.

9. FORMATION OF OMII BY OTHER SPECIES

As the small size of rat livers necessitated killing a large number of rats, it was decided to see if any other species could convert oestriol to OMII.

Ox liver slices were inactive. Whether this was due to a genuine absence of the necessary enzymes, or because of the 3 hr. period between killing the animal at the abattoir and setting up the incubations, cannot be said.

Rabbit liver slices appeared to give a better yield of OMII than rat slices and, conversely, the latter gave a better yield of 2-methoxyoestriol.

10. ISOLATION AND PRELIMINARY IDENTIFICATION

OF RABBIT OMII

a. Isolation

The same oestriol:saline:tissue ratios were
used as in the rat experiments (Section III, 4)
and the same extraction procedure was employed.
The OMII was separated from the oestriol by
chromatography on a number of sheets of 3 MM
paper in the system 70% acetic acid/ethylene
dichloride.   The OMII was eluted with methanol
and rechromatographed in the same system.   Two
batches of OMII were prepared in this way.   In
batch A, 85 mg. of OMII were obtained from 130 mg.
of oestriol and in batch B, 100 mg. of OMII from
128 mg. of oestriol (cf. rat liver, 13.5 mg. of
OMII from 300 mg. of oestriol).

Both batches were pooled.   Leaching with
ether or chloroform removed no impurities.
Ethyl acetate was then tried and the solvent
pipetted off and taken to dryness under nitrogen.
Whilst the ethyl acetate did not dissolve any
pigment it did dissolve some of the OMII.   The
OMII was then exhaustively leached with
successive 1 ml. portions of ethyl acetate.   The
combined ethyl acetate soluble material was taken

to dryness under nitrogen to give 40 mg. of pale
brown oil.   Methanol (0.2 ml.) was added to this
and allowed to stand at -17°C for 2 hr.   The
supernatant was pipetted off and the residue
dried in vacuo.   Since this did not dissolve
very readily in acetone, successive 0.1 ml.
amounts of 50% aqueous acetone were added with
warming.   Most of the solid had dissolved in
3 ml. of this solvent.   The insoluble material
was filtered off and the filtrate stood at room
temperature for 3 hr.   The solution turned
cloudy, and on standing overnight at -17°C, a
gelatinous precipitate came down.   The super-
natant was pipetted off and the residue washed
once with 0.2 ml. of cold 50% aqueous acetone.
This left 5 mg. of white residue which was dried
to constant weight in vacuo at room temperature.

b.   Reaction of OMII with Ethylene Diamine

Catecholamines can be detected on paper by
spraying with 10% (v/v) ethylene diamine in
aqueous ammonia (2 ammonia:8 water) (Weil-
Maherle and Bone, 1957).   On drying in air,
the catecholamines develop a yellow fluorescence.

This test was carried out on 25μ g. each of
OMII and oestriol and 8μ g. each of hydroquinone,

catechol, resorcinol and p-benzoquinone.   This
gives about the same equivalent weight of each
compound.

Compound	Colour	
	in air	in u.v. light
Oestriol	nil	nil
OMII	yellow	intense yellow
Catechol	yellow-brown	intense yellow
Hydroquinone	purple-brown	faint brown
Resorcinol	nil	nil
p-Benzoquinone	purple	faint brownish-yellow

With catechol, this colour is supposed to be due
to the following reaction (Weil-Malherle, 1959):-
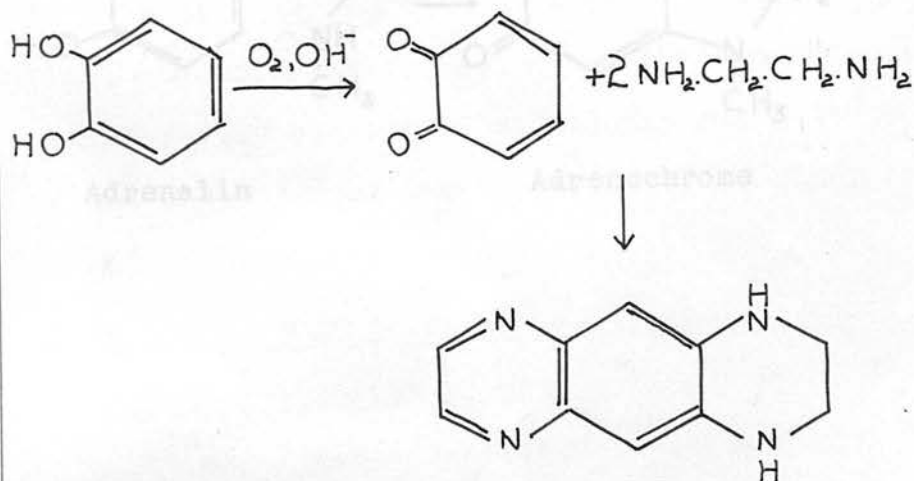
If the two para positions are blocked, as would be
the case in 2-hydroxyoestriol, only one molecule
of ethylene diamine reacts to give the following
type of compound.
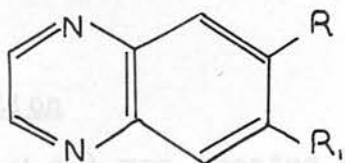
Adrenalin undergoes a similar type of oxidation
in the presence of alkali to produce adrenochrome.
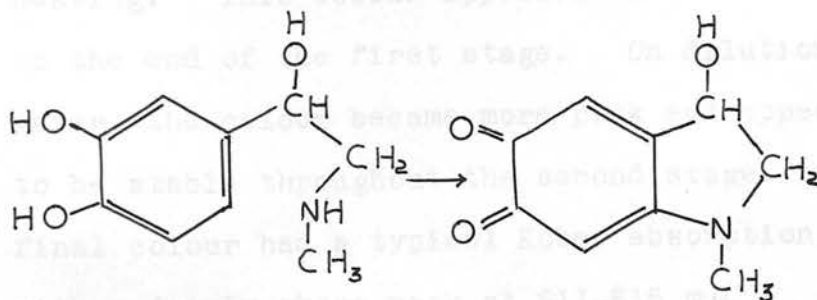
Adrenalin   Adrenochrome

OMII also turns orange in alkali.   The behaviour
of OMII in this reaction is thus compatible with
its being 2-hydroxyoestriol.   Unless steric
hindrance factors come into play, ~~1-hydroxy or~~
4-hydroxyoestriol would presumably give a similar
reaction.

c.   Kober Reaction

OMII (46 μ g.) was treated with oestriol
Kober reagent in the usual way.   In the first
stage it became pale orange within 1 min. and
then changed to a more intense orange-pink colour
which reached maximum intensity after 10 min.
heating.   This colour appeared to be stable up
to the end of the first stage.   On dilution with
water, the colour became more pink and appeared
to be stable throughout the second stage.   This
final colour had a typical Kober absorption curve
with a fairly sharp peak at 511-515 mμ.
It had about 30% of the intensity of an equal
weight of oestriol.

d.   Evidence from Co-factor Requirements for
      2-Methoxyoestriol Formation

The experiments described in Section III, 14,
indicate that DPN^+ or TPN^+, Mg^{2+}, ATP and L-
methionine are needed for the conversion of
oestriol to 2-methoxyoestriol by liver homogenates.

FIG. 7. ULTRAVIOLET ABSORPTION SPECTRUM OF OM II.

———— 115μg. OM II / 3mls. ETHANOL.

— — — 115μg. OM II / 3mls. ETHANOL + 0·1mls. N NaOH.

·········· " " AFTER STANDING FOR 30mins, 0·15mls. N HCl ADDED.
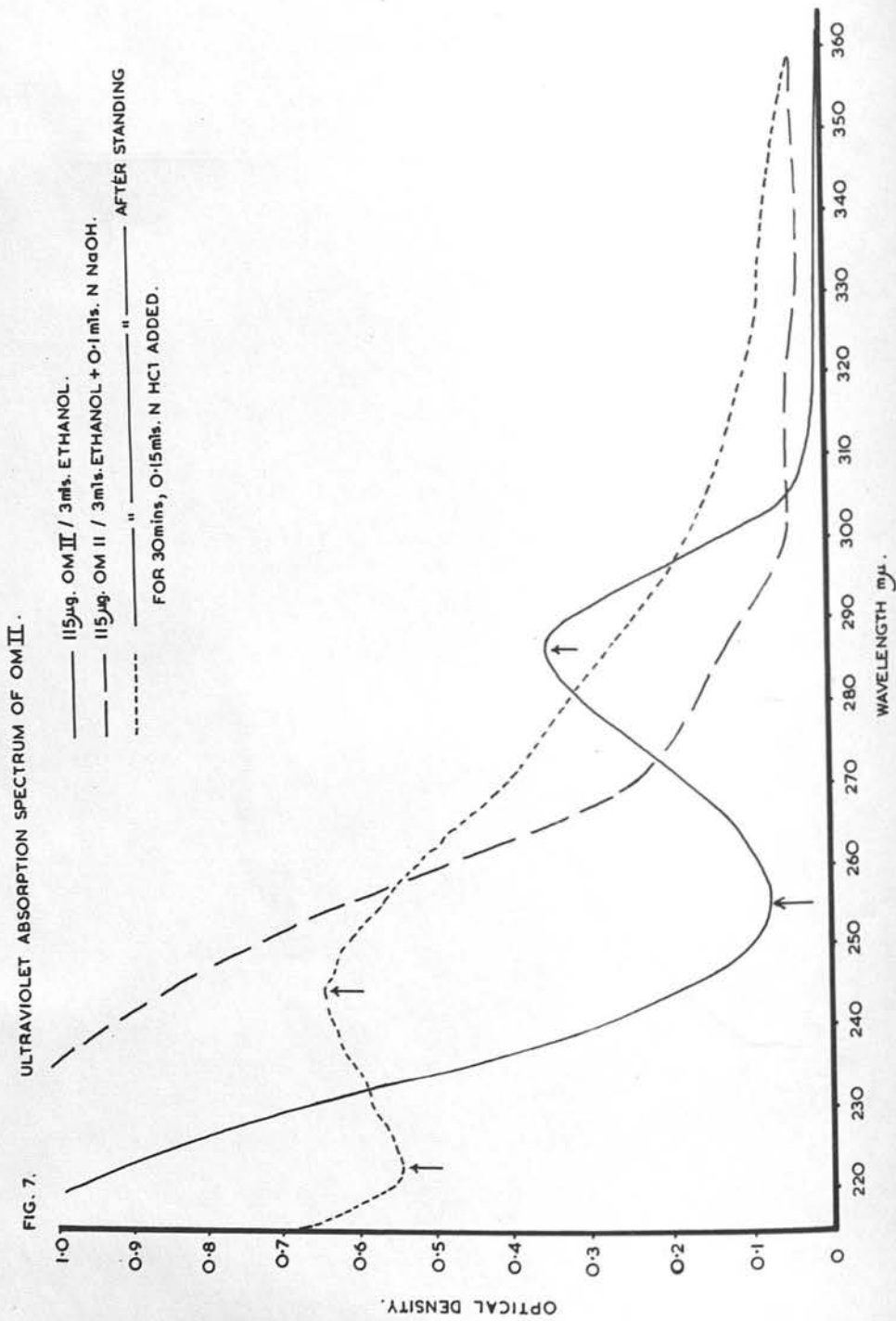
In the absence of ATP, Mg^{2+} or L-methionine the
decreased yield of 2-methoxyoestriol is balanced
by a proportionate rise in the amount of OMII
formed.   The amount of oestriol recovered is
very little affected by the absence of these
three co-factors.   In addition to this, OMII can
be converted to 2-methoxyoestriol by liver
homogenates in the presence of ATP, Mg^{2+} and L-
methionine.   All of this evidence points to OMII
being 2-hydroxyoestriol.

e.   Ultraviolet Absorption Spectrum in Neutral,
     Alkaline and Acid Ethanol

OMII (115 μ g.) was dissolved in 3 ml. of
ethanol and its u.v. absorption curve measured.
This is shown in Fig. 7.   It has a $\lambda_{min.}$
255 mμ, $\lambda_{max.}$ 287 mμ This maximum is lost
on the addition of 0.1 ml. of N-NaOH.   On the
further addition of 0.15 ml. of N-HCl, a new
maximum appeared at 245 mμ.   The spectrum in
neutral ethanol is similar to that of 2-hydroxy-
oestradiol-17β ($\lambda_{min.}$ 255 mμ, $\lambda_{max.}$ 288 mμ),
but different to that of 4-hydroxyoestradiol-17β
($\lambda_{min.}$ 254 mμ $\lambda_{max.}$ 280 mμ G.C. Mueller,
private communication).

Just what happens on the addition of alkali
is not certain, but Dr M.M. Coombs has shown
that when 2-hydroxyoestriol is dissolved in 0.1 N-
NaOH and is allowed to stand in air at room
temperature, 4 atoms of oxygen/molecule of steroid
are taken up within 10 min. and a further 1 atom
is absorbed in the course of the next hour.   The
i.r. and u.v. spectra of the product indicated
that the aromatic ring had disappeared and that
a hydroxy carboxylic acid had been formed.

f.   Infrared Spectrum

Dr Callow kindly measured the infrared
spectrum of OMII in a potassium chloride plate
and, although it was not a very good spectrum,
found that it was compatible with a structure of
2-hydroxyoestriol.

11.   ISOLATION OF CRYSTALLINE OMII AND ITS

       IDENTIFICATION AS 2-HYDROXYOESTRIOL

a. Preliminary Tests

Dr M.M. Coombs has recently synthesized
2-hydroxyoestriol by a modification of the method
used by Fishman (1958) for the synthesis of
2-methoxy oestrogens.

Its chromatographic behaviour, u.v. spectrum
in neutral, alkaline and acid ethanol, and its
reactions with Folin and Ciocalteu reagent, with
ethylene diamine and with alkali were the same
as OMII.

b. Crystallization and Melting Point Determination

A fresh batch of rabbit OMII was prepared
as before (Section III, 10, a).   From 192 mg. of
oestriol, 120 mg. of OMII were obtained as a
dark brown solid.   This was exhaustively
leached with 1 ml. portions of ethyl acetate
and the solvent taken to dryness under nitrogen.
The pale yellow solid was washed three times
with 0.5 ml. of ethyl acetate which removed most
of the contaminating pigment, leaving 19 mg. of
white solid.

This was crystallized twice from 50%
aqueous methanol at 0°C, the crystals being
washed three times with 0.1 ml. of 50% aqueous
methanol after each crystallization. This gave
1.3 mg. of white crystals which had m.p. 267-269°C
with shrinkage at 263°C (evacuated sealed tube).
2-Hydroxyoestriol had an m.p. 269-271°C
(evacuated sealed tube) using the same apparatus
and thermometer. The mixed m.p. of OMII with
2-hydroxyoestriol was 265-268°C.

c. Infrared Spectrum

The i.r. spectrum of the synthetic 2-hydroxy-
oestriol agreed very well with the spectrum of
OMII obtained by Dr Callow (10, f. above). In
particular 20 peaks in the 800-1600 cm.$^{-1}$ region
corresponded almost exactly.

It was concluded that OMII was 2-hydroxy-
oestriol.

12. DEVELOPMENT OF A METHOD FOR THE
    EXTRACTION, SEPARATION AND ESTIMATION OF
    2-METHOXYOESTRIOL, OESTRIOL AND 2-HYDROXY-
    OESTRIOL

a. Extraction Procedure

In view of the instability of OMII, this was
made as simple as possible. In experiments with
liver slices, the incubation medium was
homogenized and 5 ml. of 15% (v/v) HCl added.
With water washings, this gave a final volume of
about 20 ml. which was extracted three times with
50 ml. of ether. From the partition coefficients
between ether/water (Marrian and Sneddon, 1960),
this should extract all of the oestriol and 2-
methoxyoestriol, 90% of any 6-oxooestriol and
50% of any 6α-hydroxyoestriol. As 2-hydroxy-
oestriol has a polarity between that of 6-oxo-
and 6α-hydroxyoestriol, about 70% of this
compound should be extracted. As the extractions
are made from strongly acid solution, the amounts
extracted are probably higher than those given
above.

The ether was distilled off on a boiling
water bath, the last trace of solvent being
removed under vacuum.

In experiments not using slices, the
homogenizing step was omitted.

b. Separation of the Three Compounds

i. 2-Hydroxyoestriol

A portion of the ether extract was spotted
directly onto Whatman 3 MM paper and chromato-
graphed for about $3\frac{1}{2}$ hr. in the 70% acetic acid/
ethylene dichloride system. Standard 2-hydroxy-
oestriol was run on each paper. The strip
containing the standard material was cut off and
sprayed with Folin and Ciocalteu reagent to
locate the 2-hydroxyoestriol. The corresponding
areas of the unsprayed portion of the paper were
eluted with methanol. For this a device
designed by Saffran and Sharman (1960) was used
which enabled most of the steroid to be eluted
with 2 ml. of methanol. The methanol solution
was taken to dryness under nitrogen.

After cutting out the 2-hydroxyoestriol
areas, the rest of the paper was sprayed with
Folin and Ciocalteu reagent to check that all of
the 2-hydroxyoestriol area had been cut out and
also to check that this area was not contaminated
with oestriol.

ii.   2-Methoxyoestriol and Oestriol

During the isolation of 2-methoxyoestriol
(Section III, 5, a) it behaved in a similar
manner to 16-epioestriol in all the partition
methods used.   As a preliminary procedure it
was decided to test a method based on the
separation of oestriol and 16-epioestriol.   The
residue from the ether extraction was dissolved
in ethylene dichloride and partitioned on celite
columns using the system 70% methanol/ethylene
dichloride (Bauld, 1955, see Section I, 2, h).
The first 4 ml. of eluate were collected as cut A,
the next 7 ml. as cut B, and the next 25 ml. as
cut C.   The column was then stripped with 15 ml.
of methanol (cut D).   Half of each cut was
chromatographed on Whatman no. 1 paper in the
70% acetic acid/ethylene dichloride system for
3 hr.   Cut A contained no phenols, but B
contained all of the 2-methoxyoestriol but no
oestriol.   Cut C contained only oestriol and
cut D no phenols.   This looked promising, but
when the other half of cut B was estimated for
2-methoxyoestriol with Kober reagent (Section I,
2, g), the final colour was unreadable due to
charring.   This was caused by tissue pigment

eluted from the column in the 2-methoxyoestriol
fraction.

During the isolation of 2-methoxyoestriol
(Section III, 6, b), most of the contaminating
pigment was removed by alumina chromatography
and this was adapted for use in the estimation
procedure.   An alumina column (0.7 x 6 cm.),
deactivated with 2% of water, was prepared in
benzene.   A mixture of 2-methoxyoestriol and
oestriol was dissolved in benzene and transferred
to the column.   Each cut was chromatographed in
the formamide/chloroform system for 11 hr.   The
pigment stayed as a greenish band at the top of
the column throughout the elution.   No steroid
was eluted with 20 ml. of 1% methanol in benzene.
The first 20 ml. of 3% methanol in benzene eluted
all of the 2-methoxyoestriol and a small amount
of oestriol.   The next two 20 ml. cuts both
contained oestriol.   Unfortunately, the
procedure does not separate 2-methoxyoestriol and
oestriol, but it removes the pigment very
effectively.   To cut down the apparent tailing
of the oestriol, the following method was finally
adopted for the alumina stage of the separation.
The columns were prepared as before, the initial

20 ml. of 1% methanol in benzene being discarded.
The eluting solvent was then changed to 4%
methanol in benzene and the next 50 ml. collected
as the combined 2-methoxyoestriol and oestriol
fraction.   This was evaporated to dryness under
an air blast.

This apparently complex procedure was used
in preference to the usual phenol separation
because of the incomplete extraction of 2-methoxy-
oestriol from chloroform by N-NaOH (Section II, 7,
b).

When this residue was chromatographed on a
celite column as described above, the 2-methoxy-
oestriol fraction contained no pigment and gave
the normal 2-methoxyoestriol Kober reaction.

The experiments on the stability of OMII
(Section III, 8, c) had shown that this compound
was not eluted from alumina columns under these
conditions.

To test the recovery of 2-methoxyoestriol
when taken through this separation procedure,
20 μ g. of 2-methoxyoestriol were added to 400
mg. of liver slices plus 2 ml. of Krebs'
phosphate saline, and immediately processed as
above.   Recovery was 101%.   At the 150 μ g.
level the oestriol recovery was 70-75%.

c.   Estimation

    i.   Oestriol.   Estimated by Kober reaction
(Section I, 2, g).

    ii.   2-Methoxyoestriol.   As described in
Section III, 6, g, this compound developed a
red colour after heating for 2 min. in the Kober
reaction.   This colour faded towards the end of
the first stage and the final colour was only
20% that of an equivalent amount of oestriol.
The characteristics of this initial red colour
were investigated.

a.   Absorption Spectrum.   2-Methoxyoestriol
(15μ g.) plus 3.5 ml. of Kober reagent were heated
in a boiling water bath for 5 min.   No hydro-
quinone was added.   The tubes were cooled in
cold water and the absorption spectrum of the
colour so developed was measured.   The curve
showed a maximum at 522 mμ as shown in Fig. 6.

b.   Time of Heating.   By varying the time of
heating it was found that the colour reached
maximum intensity after 4 min. heating, faded
slightly up to 8 min. and then more rapidly up
to 20 min. heating.   The colour which had
developed after 4 min. heating was stable at
room temperature for at least 20 min.

c. Reproducibility. Good duplicate estimates
were obtained and the colour obeyed Beer's Law
up to a concentration of 50μ g./3.5 ml. of
reagent.

d. Final Method. The dry residue was heated
with 3 ml. of oestriol Kober reagent for 4 min.
in a boiling water bath. The tubes were then
cooled in a cold water bath and the optical
density at 522 mμ measured. The amount of 2-
methoxyoestriol/tube was then found by reference
to a calibration graph. At the 7μ g. level,
this method gave an estimate to within $\pm4\%$ (see
Appendix 1).

iii. 2-Hydroxyoestriol. In the preliminary
experiments with crude 2-hydroxyoestriol, it
appeared to behave in a similar way to 2-methoxy-
oestriol in the Kober reaction. Unfortunately,
when the red colour which developed after 10 min.
heating in a boiling water bath was further
investigated, it was found to be unsuitable as
an estimation procedure. It did not give good
duplicate values and the colour did not obey
Beer's Law very well.

2-Hydroxyoestriol was first detected
because of its behaviour with Folin and Ciocalteu's

reagent (Section III, 3).   This reagent has been

used by Davies and Mitchell (1954) to estimate

oestrogens, and this method was then tested with

2-hydroxyoestriol.   Preliminary experiments were

carried out with oestriol.

Oestriol (5μ g.) was incubated at 37°C for

15 min. with 0.5 ml. of Folin and Ciocalteu

reagent plus 0.5 ml. of 1 M-sodium carbonate.

The absorption spectrum of the blue colour so

produced was measured in a 2 cm. light path cell.

There was a single maximum at 745 mμ.   When a

small amount of 2-hydroxyoestriol was treated in

a similar way, the colour had a flat maximum at

740-760 mμ.

In the final method, the dry residue was

incubated at 37°C for 20 min. with 1.5 ml. of

Folin and Ciocalteu reagent plus 1.5 ml. of 1 M-

sodium carbonate.   The final colour was read in

a 1 cm. light path cell at 745 mμ.   The readings

so obtained did not obey Beer's Law exactly but

this discrepancy was not serious up to a

concentration of about 10 μ g./3 ml. of final

volume.   At the 10 μ g. level this method gives

an estimate to within ±2.54% (see Appendix 1).

The main disadvantage of this method is its

lack of specificity.

13. OESTRIOL METABOLISM BY RAT LIVER SLICES

Using the method described above, the
following results were obtained.

All incubation tubes contained 400 mg. of
adult male rat liver slices plus 4 ml. of Krebs'
phosphate saline, pH 7.4, plus 200 μ g. of
oestriol in 0.05 ml. of PG.   The tubes were
incubated under oxygen for 2 hr. at 37°C.

	Oestriol recovered %	2-Methoxy- oestriol formed %	2-Hydroxy- oestriol formed %	Total recovery %
Slices boiled 1 min. prior to incubation	70	1	0	71
Viable slices. Oestriol added just prior to extraction	70	0	0	70
Viable slices	5	10	18	33

It can be seen that about 40% of the added
oestriol is unaccounted for.   To check that some
of this loss was not due to glucuronide formation,
the following additional extraction was carried·
out.   The aqueous phase remaining after the
free oestrogens had been extracted was extracted
once with 50 ml. of ether and the ether discarded.

This was to ensure that all the free oestrogens had been extracted. The aqueous phase was extracted twice with 50 ml. of n-butanol to extract any conjugates, and the butanol evaporated to dryness under vacuum. The residue was dissolved in 5 ml. of methanol and a 4 ml. portion transferred to a conical flask and evaporated to dryness under nitrogen. To each flask was added 5 ml. of 1 M-acetate buffer, pH 4.6, and 5 x 10^3 Fishman units of soluble limpet β-glucuronidase. This limpet powder also contains a sulphatase. The flasks were stoppered and incubated at 37°C for 48 hr. Each flask was extracted three times with 10 ml. of ether and the ether evaporated to dryness under nitrogen. The residue was estimated for 2-methoxyoestriol, oestriol and 2-hydroxyoestriol but none could be detected, so it can be assumed that conjugation is not a significant metabolic pathway in this system.

14. OESTRIOL METABOLISM BY RAT LIVER

   HOMOGENATES. COFACTOR REQUIREMENTS FOR

   2-METHOXYOESTRIOL FORMATION

The experiments on the formation of 2-
methoxyoestriol were carried out first because
at that time the structure of OMII was not known
with any certainty. It was reasoned that by
studying the cofactor requirements for the
synthesis of 2-methoxyoestriol some evidence
would be produced to show whether or not OMII
was 2-hydroxyoestriol.

a. Preliminary Experiments

   i. Choice of Buffer System

In other cases of O-methylation (Axelrod
and Tomchick, 1958; Braithwaite and Goodwin,
1958; Pellerin and D'Ionio, 1958) it has been
shown that adenosyl methionine can be the
methyl donor and that this methylase requires
Mg^{2+}. Cantoni (1953) has shown that a high
concentration of Mg^{2+} is needed (0.2 M for
optimal synthesis) for the formation of adenosyl
methionine from ATP and methionine. However,
because of the low solubility of magnesium
phosphate, it is rather difficult to attain a

high Mg^{2+} concentration in a phosphate buffer at
pH 7.4.

In order to discover the best buffer system
to use, and working on the assumption that
methoxylation occurs by hydroxylation and
subsequent methylation by adenosyl methionine,
the following preliminary experiment was carried
out.

Each incubation tube contained the following:-

1 ml. of 10% male rat liver homogenate in

0.25 M-sucrose plus 0.12 M-nicotinamide

0.1 ml. of 0.045 M-sodium lactate

0.1 ml. of 0.045 M-glucose-6-phosphate

0.5 mg. of DPN^+

0.5 mg. of TPN^+

0.3 ml. of 0.025 M-ATP, pH 7.4

0.2 ml. of 0.3 M-L-methionine, pH 7.4.

To tubes 2, 3 and 4, 200 μg. of oestriol in
0.05 ml. of PG were added.

To tubes 1 and 4 were added 0.33 ml. of 0.04
M-potassium phosphate, pH 7.4, plus 0.33 ml. of
0.5 M-potassium chloride plus 0.33 ml. of 0.048 M-
magnesium sulphate (Brownie and Grant, 1956).

To tube 2 was added 1 ml. of 0.35 M-Tris
(2-amino-2-hydroxymethyl-1,3-propanediol) HCl
buffer, pH 7.4, plus 0.2 ml. of 2 M-magnesium
chloride.

To tube 3 was added 1 ml. of Krebs' phosphate
saline, pH 7.4.

The tubes were incubated under oxygen for 2
hr. at 37°C. They were extracted as before
(Section III, 12, a) and the residue chromato-
graphed on 3 MM paper in the 70% acetic acid/
ethylene dichloride system for 3 hr.

Tube 2 contained a large amount of oestriol,
a trace of 2-methoxyoestriol and no 2-hydroxy-
oestriol.

Tube 3 had a trace of 2-methoxyoestriol and
a very pronounced 2-hydroxyoestriol spot. There
was less oestriol than in tube 2.

Tube 4 appeared to contain about 20 μ g. of
2-methoxyoestriol but less of the 2-hydroxy
compound than in tube 3. There was also less
oestriol.

Consequently the potassium chloride/Mg^{2+}/
potassium phosphate buffer was used in this
study. The ability of this buffer, but not the
Tris/HCl/$MgCl_2$ one, to support oestriol
metabolism was not further investigated. It is

interesting that Grant and Brownie (1955) found that Tris-HCl was better than phosphate buffer for the study of 11β-hydroxylation by ox adrenal mitochondria.

In view of Lipmann's demonstration (1958) that active sulphate can be synthesized from ATP and inorganic sulphate in the presence of rat liver supernatant enzymes, the magnesium sulphate was replaced by magnesium chloride. This cuts out an alternative pathway for ATP metabolism and also excludes the possibility of oestriol sulphate formation.

ii.   Choice of Cell Fraction

In order to use as simple a system as possible in this study, the following cell fractions were tested:- homogenate minus debris and nuclei, mitochondria, mitochondria plus microsomes, microsomes plus supernatant, microsomes, supernatant, mitochondria plus supernatant.   In addition to ATP and L-methionine, a DPNH and TPNH producing system was added to each incubation.

There was no 2-hydroxyoestriol in the mitochondria or supernatant incubations, but it was detectable in all the other tubes.   The

largest amounts were present in the tubes
incubated with whole homogenate and with the
microsome plus supernatant fraction.   There were
about 5 µg. of 2-methoxyoestriol in the whole
homogenate incubation and a trace in the one with
microsomes plus supernatant.   In no case was
there a reasonable synthesis of 2-methoxyoestriol
with any of the cell fractions, so it was
decided to use whole homogenates.   To increase
the enzymic activity, 20% homogenates in 0.25
M-sucrose were used.

    iii. Optimal Incubation Time

    Each incubation tube contained the
following:-

        1 ml. of 20% male rat liver homogenate

        in 0.25 M-sucrose

        0.1 ml. of 0.045 M-sodium lactate

        0.1 ml. of 0.045 M-glucose-6-phosphate

        0.5 mg. of DPN^+

        0.5 mg. of TPN^+

        0.3 ml. of 0.025 M-ATP

        0.2 ml. of 0.3 M-L-methionine, pH 7.4.

To all tubes except no. 1 were added 1 ml. of
potassium phosphate/magnesium chloride/potassium
chloride buffer, pH 7.4, and 120 µg. of oestriol
in 0.03 ml. of PG.   The volume was made up to

3 ml. with water.   The incubations were carried

out under oxygen at 37°C.

Incubation Time hr.	Oestriol Recovered %	2-Methoxyoestriol Formed %
0	–	0
0.5	13	16
1	13	20
2	14	17
4	14	16

2-Hydroxyoestriol was not estimated.   Only one

estimation was made.

iv. Final Incubation Conditions

Not counting the various cofactors to be

tested, the following incubation system was used:-

1 ml. of 20% homogenate of male rat

liver in 0.25 M-sucrose

0.33 ml. of 0.5 M (240 μ moles KCl)

0.33 ml. of 0.04 M (192 μ moles)

potassium phosphate, pH 7.4

150 μ g. (1.95 μ moles) oestriol

in 0.035 ml. of PG.

The final volume was made up to 3 ml. with water

in all cases.   The incubations were carried out

FIG. 8A.   OESTRIOL METABOLISM BY RAT LIVER HOMOGENATES.   EFFECT OF DPN⁺ AND TPN⁺.

ALL INCUBATIONS CONTAINED 150 μM. MgCl₂ + 5 μM. ATP + 60 μM. L-METHIONINE.
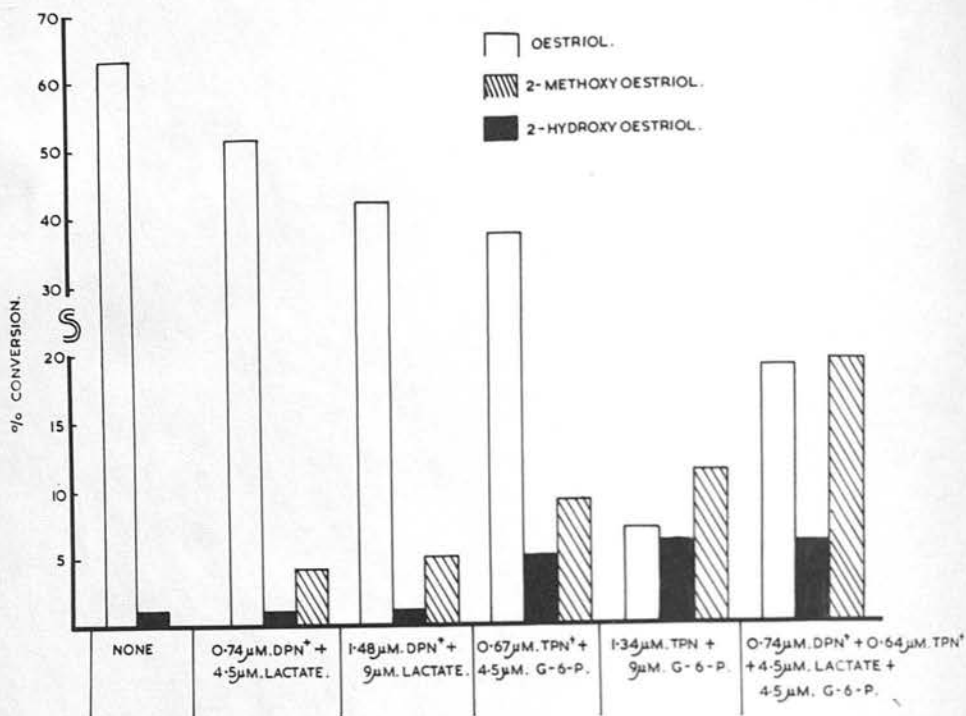
FIG. 8B.   OESTRIOL METABOLISM BY RAT LIVER HOMOGENATES.   EFFECT OF MAGNESIUM, ATP
           & L-METHIONINE.

ALL INCUBATIONS CONTAINED 0·74 μM. DPN⁺ + 0·64 μM. TPN⁺.
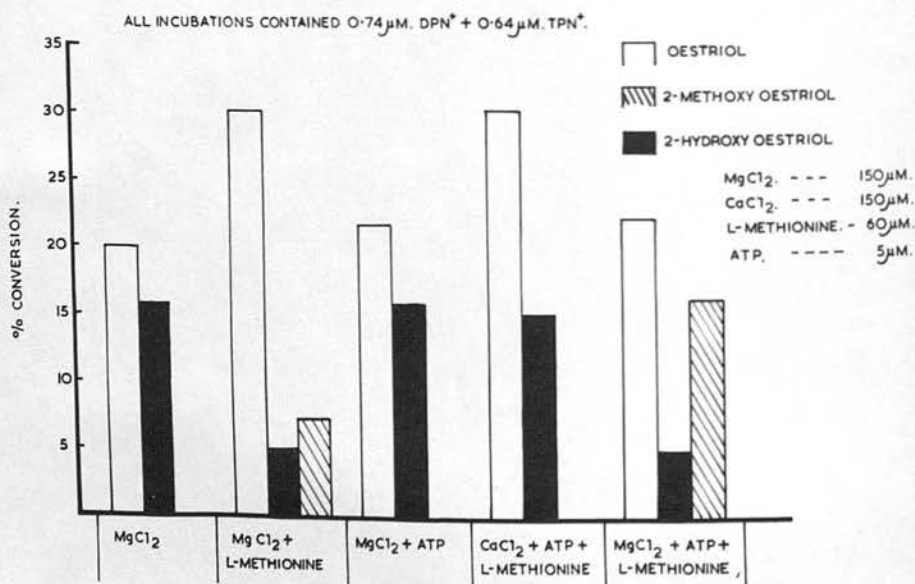
in stoppered Q. and Q. test tubes for 1 hr. under oxygen at 37°C with shaking.

The ether soluble residue was dissolved in 3.3 ml. of methanol and a 2 ml. portion used for 2-methoxyoestriol and oestriol estimation and a 1 ml. portion for 2-hydroxyoestriol determination.

In all the experiments an extraction control was obtained by adding 150 μ g. of oestriol to the complete incubation medium just prior to extraction. The 2-methoxyoestriol and 2-hydroxy-oestriol values for these control incubations were used as blank values and subtracted from the values obtained in the other tubes. Regular 70-75% recoveries of the added oestriol were obtained.

b. Effect of Adding DPN[+] and TPN[+]

This is shown in Fig. 8a. In the absence of DPN[+] or TPN[+], the amount of oestriol metabolized is small and very little, if any, 2-hydroxy- or 2-methoxyoestriol is formed. Graubard and Pincus (1944) have suggested that oestrogens might be metabolized via a phenolase type oxidation. They demonstrated that oestradiol-17β could be oxidized to unknown

products by potato tyrosinase, but not by rabbit liver preparations. However, the results shown here would seem to rule out this type of metabolic pathway. In this connection it is noteworthy that Kaufman (1959) has shown that TPNH is required for the conversion of phenyl alanine to tyrosine in the liver.

The additive effect of $DPN^+ + TPN^+$ would suggest the presence of two separate enzymes for the 2-hydroxylation step. Experiments on the synthesis of 2-hydroxyoestriol by microsome plus supernatant fraction (Section III, 16, b) do not confirm this.

c. Effect of Ca^{2+}, Mg^{2+}, ATP and L-Methionine

The results are shown in Fig. 8b. It can be seen that Mg^{2+}, ATP and L-methionine are required for the optimal synthesis of 2-methoxy-oestriol. No other methyl donors were tested. Whether the Mg^{2+} is required solely for the formation of adenosyl methionine or for the methylation step as well, cannot be said from this experiment. By analogy with other O-methylations (see Section II, 14, a) it would seem likely that the Mg^{2+} is needed for both reactions.

The small synthesis of 2-methoxyoestriol in the absence of ATP was rather unexpected. This is presumably due to the presence of endogenous ATP (or an ATP synthesizing system). Dialysis of the homogenate was not tested, and pre-incubation of the homogenate could not be used because of the lability of the methylating system (see below).

It would appear that Mg^{2+} and ATP are not required for the formation of 2-hydroxyoestriol. The results also indicate that ATP has a slight stimulating effect on the amount of oestriol metabolized, and that Ca^{2+} produces a small inhibition. This will need further study as other explanations are possible.

d. Preincubation of the Homogenate

Where stated, the homogenate was pre-incubated for 30 min. at 37°C in air. All the incubation tubes contained the DPN^+, TPN^+, G-6-P, sodium lactate, L-methionine and buffer concentrations used for optimal 2-methoxyoestriol synthesis (see above).

Additions	Oestriol Recovered %	2-Methoxy- oestriol %	2-Hydroxy- oestriol %	Total %
Extraction control	72	–	–	72
1 ml. homogenate	14	3	14	31
1 ml. homogenate + 5 μ M ATP	10	15	8	33
1 ml. preincubated homogenate	41	0	10	51
1 ml. preincubated homogenate + 5 μ M ATP	19	0	11	30

Obviously the methylating system has been
destroyed by this preincubation treatment.   It
does, however, support the suggestion made in
section c above that ATP has no effect on the 2-
hydroxylase, but does stimulate oestriol
metabolism.

e. The Conversion of 2-Hydroxyoestriol to
    2-Methoxyoestriol

These experiments were only carried out
qualitatively.   The ether soluble extracts of
the incubations were chromatographed for 11 hr.
in the formamide/chloroform system.

When 2-hydroxyoestriol was incubated with
the complete methoxylating system there was a
20-30% conversion (as determined by the intensity
of the Folin and Ciocalteu spots) to 2-methoxy-
oestriol.   There was also a suggestion of a blue
spot slightly more polar than 16-epioestriol.
This might be the 2-hydroxy-3-methoxy compound.

The omission of DPN^+ and TPN^+ had no effect
but in the absence of L-methionine, no 2-methoxy-
oestriol was formed.

It would thus appear that 2-methoxyoestriol
can be synthesized in the liver by the following
pathway:-

$$\text{oestriol} \xrightarrow[\substack{O_2}]{\text{DPNH or TPNH}} \underset{\text{oestriol}}{\text{2-hydroxy-}} \xrightarrow[\text{L-methionine}]{\text{ATP, Mg}^{2+}} \underset{\text{oestriol}}{\text{2-methoxy-}}$$
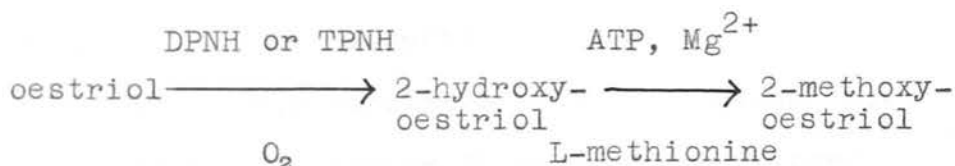
The conclusion that it is the reduced pyridine
nucleotide which is required comes from the
study of 2-hydroxylation with microsome plus
supernatant fraction (Section III, 16, c).

15. THE METHOXYLATION OF OESTRONE,

     OESTRADIOL-17β, 17α-ETHINYLOESTRADIOL-17β

     AND STILBOESTROL

To see if these compounds could be
methoxylated, two incubation tubes were set up
for each compound. The complete methoxylating
system described in Section III, 14, c, was added
to tube A of each pair, whilst no L-methionine
nor ATP was added to the B series. To each tube
150 μ g. of the required compound in 0.025 ml. of
methanol were added. The incubation and
extraction were as before (Section III, 14, b).
The ether soluble residue of each incubation was
divided into three parts.

Part 1 was chromatographed on Whatman no.
42 paper in the system 70 methanol:30 water/
50 benzene:50 hexane for $3\frac{1}{2}$ hr. 2-Methoxy-
oestrone, oestrone and oestradiol-17β were run as
standards. This system would separate any
ortho methoxy derivative from the parent phenol,
but the corresponding 2-hydroxy compound would
not migrate from the origin.

In all of the A series except that of
oestradiol-17β, there was a Folin and Ciocalteu

positive spot which was less polar than the
original compound.   In the case of oestrone,
this compound had the same mobility as authentic
2-methoxyoestrone.   In the B series these less
polar metabolites were absent in all cases.

In the A tube of the oestradiol-17β
incubation, two compounds having the same
mobility as 2-methoxyoestrone and oestrone could
be detected, but not apparently any 2-methoxy-
oestradiol-17β.   It may be that this compound
has the same mobility as oestrone in this system.

The R_F values of the various compounds are
given below.   With stilboestrol the situation is
complicated by the fact that this chromatographic
system appears able to separate the _cis_- and _trans_-
isomers.

Compound	R_F	Compound	R_F
Oestradiol-17β	0.27	Oestrone	0.57
17α-Ethinyl-oestradiol-17β	0.37	'2-Methoxy-17α-ethinyl-oestradiol-17β'	0.60
Stilboestrol a	0.25	'Methoxy-stilboestrol'	0.60
Stilboestrol b	0.43	2-Methoxyoestrone	0.73

Part 2 was chromatographed for 10 hr. in the formamide/chloroform system. No compound more polar than oestradiol-17β was detectable in either of the oestrone incubations. In both of the oestradiol-17β tubes there were about 3μ g. of a compound with the same mobility as oestriol. This is probably 6α-hydroxyoestradiol-17β. In the B but not in the A oestradiol-17β incubation there was a spot with the same mobility as 2-hydroxyoestradiol-17β which turned blue with Folin and Ciocalteu reagent in the absence of alkali. The authentic 2-hydroxyoestradiol-17β had a polarity intermediate between that of 16-epioestriol and oestriol.

In the B tube of the 17α-ethinyloestradiol-17β incubation, and in both stilboestrol tubes, there were metabolites slightly more polar than 16-epioestriol which, from their behaviour with Folin and Ciocalteu reagent, could have been the o-hydroxylated compound. With stilboestrol, the B tube contained about twice as much as the A tube of this metabolite.

In this system, 17α-ethinyloestradiol-17β and stilboestrol migrate at about the same rate as oestradiol-17β.

Part 3 was chromatographed in the system
70% acetic acid/ethylene dichloride for $4\frac{1}{2}$ hr.
No compound more polar than oestriol could be
detected in any of the tubes.

As a preliminary experiment, this indicated
that oestrone, 17a-ethinyloestradiol-17β and one
of the stilboestrol isomers could be ortho-
methoxylated.   Oestradiol-17β can apparently be
2-hydroxylated, but the subsequent methylation is
open to doubt.

16. OESTRIOL METABOLISM BY RAT TISSUE
     PREPARATIONS.   COFACTOR REQUIREMENTS FOR
     THE SYNTHESIS OF 2-HYDROXYOESTRIOL

a. Preliminary Experiments

     i. Choice of a Suitable Buffer System

A simple potassium phosphate buffer, pH 7.4,
supported a 20% conversion of oestriol to 2-
hydroxyoestriol, so this was used in preference
to the potassium phosphate/potassium chloride/
magnesium chloride system used previously. The
same final concentration of phosphate was used
in both cases - i.e. 192 μ moles of phosphate/
3 ml. of incubation medium.

ii. Extraction and Separation of Steroids

The extraction was as used previously in
Section III, 14. The ether soluble residue was
dissolved in 2.5 ml. of methanol and a 1 ml.
portion used to estimate 2-hydroxyoestriol.
Another 1 ml. portion was taken to dryness on a
boiling water bath with the aid of an air blast
and then estimated for oestriol after
chromatography on a celite column with the
system 70% methanol/ethylene dichloride. This
method thus differed from that used in the 2-
methoxyoestriol experiments in that the alumina
chromatography was omitted. This resulted in
about 10% higher recoveries of oestriol in the
extraction controls.

To check that no 2-hydroxyoestriol was
passing through this procedure with the oestriol,
a mixture of oestriol and 2-hydroxyoestriol was
put through this separation method. Instead of
estimating the final oestriol fraction it was
chromatographed in the 70% acetic acid/ethylene
dichloride system for 4 hr. The only phenol
present was oestriol.

iii.   Metabolism of Oestriol by Ovary, Uterus,
Kidney and Liver Homogenates.   The Demonstration
of a Sex Difference in the Liver 2-Hydroxylase
Activity.

To investigate the distribution of the 2-
hydroxylase and to find which was the best tissue
to use in the cofactor studies, the following
experiment was performed.   Each incubation tube
contained the following:-

      1 ml. of 20% homogenate in 0.25 M-sucrose

      1 ml. of 0.192 M-potassium phosphate,

        pH 7.4

      100 μ g. of oestriol in 0.025 ml. of PG

      0.1 ml. of 0.045 M-sodium lactate

      0.1 ml. of 0.045 M-glucose-6-phosphate

      0.5 mg. of DPN$^+$

      0.5 mg. of TPN$^+$

Incubations were for 1 hr. under oxygen at 37°C.

Tissue	Oestriol Recovered %	2-Hydroxy-oestriol Formed %	Total Recovery %
Female liver (extraction control)	81	-	81
Female liver	25	22	47
Male liver	35	12	47
Female kidney	37	1	38
'Normal' uterus	76	1	77
Ovary	70[X]	1[•]	71

X only one estimation

• only two estimations

Unfortunately the oestrus state of the female rats was not determined. The uteri appeared to be in the resting state as judged by their size and weight.

The enhanced formation of 2-hydroxyoestriol with female liver might be due to an increased amount of enzyme, or to an elevated amount of some unknown activator. Huggins and Yao (1959)

have shown that oestradiol-17β increases the
hepatic glucose-6-phosphate dehydrogenase and
lactic dehydrogenase activities of ovariectomized
rats, and this might explain this increased 2-
hydroxylase activity.   It is hoped to clarify
this point in the near future.

The metabolism of oestriol by kidney
preparations is described in more detail in
Section II, 9.   This organ contains a 16-hydroxy
steroid dehydrogenase which can oxidize oestriol
to 16-oxooestradiol-17β.

Female rats were used in the future
experiments.

iv.   Choice of Cell Fraction

Before the detection of 2-hydroxy- and 2-
methoxyoestriol, a series of experiments had been
carried out on the metabolism of oestriol by
liver cell fractions (see Appendix II, 5).   The
extraction procedure described in Section II, 3,
was used, and consequently only the oestriol
recovered was measured.   These experiments
indicated that the pyridine nucleotide dependent
metabolism of oestriol was localized in the
microsomal and supernatant fractions.   The
qualitative experiment described in Section III,

14, a, indicated that the 2-hydroxylase is located
in the microsomal fraction.

Using the incubation conditions described in
iii. above, the following results were obtained:-

Liver Preparation	Oestriol Recovered %	2-Hydroxy- oestriol Formed %	Total Recovery %
Whole homogenate. No oestriol added.	0	-	0
Whole homogenate. Oestriol added	6	30	36
Homogenate minus cell debris and nuclei	9	30	39
Microsome plus supernatant fraction	24	17	41

Only one set of duplicate estimations was carried
out in this experiment.   It can be seen that the
absence of mitochondria has very little effect on
the total recovery of steroid, but the 2-
hydroxylase activity dropped appreciably.   As
mitochondria do not possess a 2-hydroxylase
(Section II, 14, a), they may be influencing the

reaction _via_ some unknown cofactor.

As there was an appreciable formation of
2-hydroxyoestriol with the microsome plus super-
natant fraction this was used in the following
experiments.

v. Effect of Adding Oxidizable Substrates

In the 2-methoxyoestriol experiments it was
found that no exogenous TPNH- or DPNH-producing
substrate was needed (Section II, 14). An
almost similar result was noted in this
experiment. When the glucose-6-phosphate and
sodium lactate were omitted the yield of 2-
hydroxyoestriol fell slightly from 15% to 11%.

vi. Final Incubation Conditions

Each incubation tube contained 1 ml. of
0.192 M-potassium phosphate, pH 7.4, (192μ moles
of phosphate) plus 1 ml. of microsome plus
supernatant fraction from a 20% female rat liver
homogenate in 0.25 M-sucrose. Unless stated
otherwise, 0.1 ml. of 0.045 M (4.5μmoles) sodium
lactate and 0.1 ml. of 0.045 M (4.5μ moles)
glucose-6-phosphate were also added. After the
various additions, the volume was made up to 3 ml.
with water. All incubations were carried out in
stoppered Q. and Q. test tubes under oxygen at 37°C.

FIG. 9.   OESTRIOL METABOLISM BY RAT LIVER MICROSOME + SUPERNATANT FRACTION.

A)   EFFECT OF DPN⁺ AND TPN⁺.
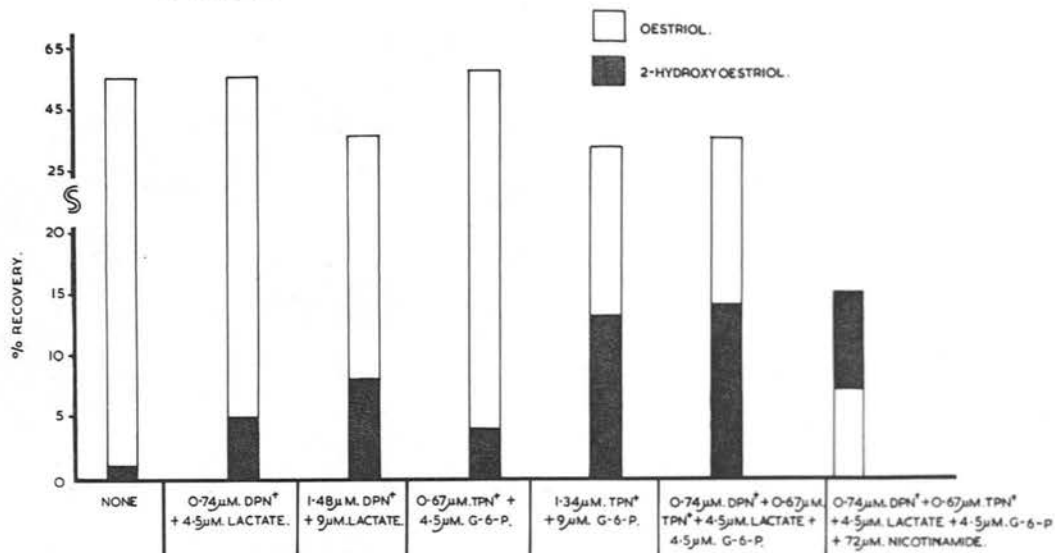
B.)   EFFECT OF PREINCUBATING THE MICROSOME + SUPERNATANT FRACTION.

ALL INCUBATIONS CONTAINED 0.74μM. DPN⁺ + 0.67μM. TPN⁺.
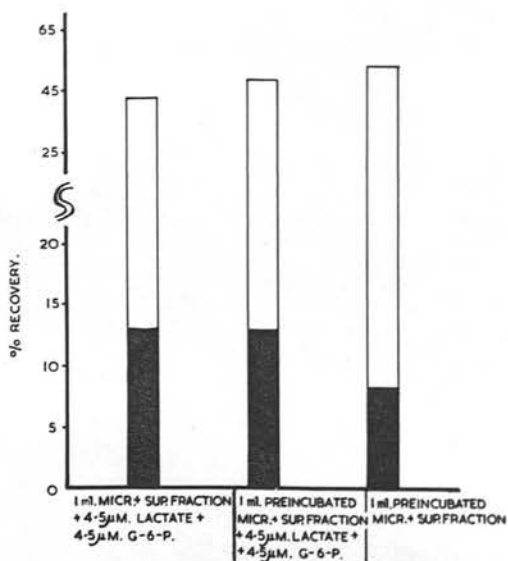
b. DPN$^+$ and TPN$^+$ Requirements

These are shown in Fig. 9a. They indicate
a surprisingly high oestriol metabolism in the
absence of added nucleotide. This is much more
noticeable than in the experiments in which
homogenates were used.(Section II, 14, b). One
major experimental difference was that male rats
were used to prepare the homogenates whilst
females were used in this experiment. In view
of the sex difference in 2-hydroxylase activity
already noted (see a, iii, above), this point
will be well worth further study.

It confirms that both DPN$^+$ and TPN$^+$ can be
used for 2-hydroxylation, but differs from the
previous experiments (Section II, 14, b) in that
the addition of 1 mg. of TPN$^+$ produces the same
result as 0.5 mg. of DPN$^+$ plus 0.5 mg. of TPN$^+$.
This could be due to the use of a more simple
incubation system in this experiment.

c. Effect of Preincubating the Microsome plus
    Supernatant Fraction

Where indicated, the microsome plus
supernatant fraction was preincubated for 10 min.
at 37°C in air. Fig. 9b shows that the 2-
hydroxylase is stable to this treatment and it
also indicates a partial requirement for an

oxidizable substrate, presumably for the
production of TPNH and DPNH.

d. Effect of Adding Nicotinamide to the
    Incubation Medium

In recent years it has become the practice
to add nicotinamide to incubation systems in
which pyridine nucleotides are used.   This
compound inhibits the enzymic destruction of
these nucleotides (Handler and Klein, 1942) so
that any reaction requiring DPN^+ or TPN^+ is
enhanced.   From Fig. 9a it can be seen that it
has no effect on the production of 2-hydroxy-
oestriol, but it had a very pronounced influence
on the amount of oestriol metabolized.   As a
result of this nicotinamide stimulation, two new
phenolic metabolites of oestriol were detected.
They are described in Section 17, below.

e. Effect of Varying the Incubation Time

All the tubes contained 72 μ moles of nicotinamide.

Incubation Time	Oestriol Recovered	2-Hydroxy-oestriol Formed	Total Recovery
hr.	%	%	%
0	82	–	82
0.5	8	12	20
1	5	12	17
1.5	4	12	16
2	3	14	17
4	3	9	12

Obviously this nicotinamide stimulated metabolism is very rapid.   It would also suggest that 2-hydroxyoestriol is stable under these conditions.

17. PRELIMINARY EXPERIMENTS ON THE
     NICOTINAMIDE STIMULATED METABOLISM OF
     OESTRIOL BY RAT LIVER MICROSOME PLUS
     SUPERNATANT FRACTION

a. Detection of Two New Metabolites

     All of the unused residues from the time
course experiment were combined and taken to
dryness under vacuum. The residue was dissolved
in 1 ml. of ethanol plus 1 ml. of glacial acetic
acid and subjected to a Girard separation. The
bicarbonate wash was replaced by an additional
water wash in both the ketonic and non-ketonic
fractions. A portion of each fraction was
chromatographed in the formamide/chloroform
system for 5 hr. and on no. 1 paper in the 70%
acetic acid/ethylene dichloride system for 4 hr.

     i. Non-Ketonic Fraction

     This contained only oestriol and 2-hydroxy-
oestriol. Even although standard 6α-hydroxy-
oestriol had migrated nearly one-third of the
way down the paper in the acetic acid system,
there was no corresponding spot in the non-
ketonic fraction.

ii. Ketonic Fraction

This indicated the presence of two previously
undetected metabolites.   The less polar one
(OMIV) had the same mobility as 16-oxooestradiol-
17β in the formamide/chloroform system.   The
more polar one (OMIII) had the same mobility as
oestriol in the formamide/chloroform system, but
was slightly more polar than oestriol in the
acetic acid system.   OMIII developed the Folin
and Ciocalteu blue colour in the absence of
ammonia, which suggested that it might be related
to 2-hydroxyoestriol.   The fact that the yield
of 2-hydroxyoestriol is unaffected by nicotinamide
or by the length of incubation would suggest that
OMIII is not a metabolite of 2-hydroxyoestriol.

The remainder of the ketonic fraction was
chromatographed in the formamide/chloroform
system for 5 hr. and the areas corresponding to
OMIII and OMIV were eluted with methanol.   The
solutions were taken almost to dryness under
nitrogen and the residual formamide removed by
extracting the steroids into ether.

b. Preliminary Identification of OMIV

i. Fast Black Salt K Formation

Half of the material was converted to the Fast Black Salt K derivative. It had the same mobility as the derivative of authentic 16-oxo-oestradiol-17β in the system 30 ethanol:70 water/ 2 toluene:1 petroleum ether.

ii. Sodium Borohydride Reduction

The remainder of the OMIV was dissolved in 1 ml. of methanol and reduced with 2 mg. of sodium borohydride. As judged from their chromatographic behaviour, the main reduction product was 16-epioestriol plus a trace of oestriol. This is the same as the sodium borohydride reduction of 16-oxooestradiol-17β (Loke, 1958).

It thus seems probable that OMIV is 16-oxo-oestradiol-17β.

c. Possible Structure of OMIII

The most likely compound is 2-hydroxy-16-oxooestradiol-17β. Another possibility is that it is the o-quinone derivative of 2-hydroxyoestriol but this seems unlikely because of the fact that OMIII does not strongly absorp u.v. light and also because the yield of 2-hydroxyoestriol from

oestriol does not vary significantly with incubation time.

18. DISCUSSION

This hydroxylation at the 2-position of oestriol is the first in vitro demonstration of enzymic attack on the benzenoid A ring of an oestrogen, and it poses some interesting questions as to the mechanism of such an hydroxylation and as to the possible further metabolism of this catechol.

It has been shown here that the cofactor requirements for this hydroxylation are, as far as can be determined, the same as those for other steroid hydroxylations, namely reduced pyridine nucleotide and oxygen. This case is interesting in that either DPNH or TPNH can be utilized. Steroid hydroxylation appears to be a very complex process. Tomkins, Curran and Michael (1958) have shown that for the 11β-hydroxylation of deoxycorticosterone by adrenal mitochondria, three enzymes are needed as well as a heat stable cofactor other than TPNH and oxygen. An

interesting link between the aliphatic steroid hydroxylations and hydroxylation of the A ring of oestrogens is provided by Kaufmann's work (1959) on the _in vitro_ conversion of phenyl alanine to tyrosine. He found that in addition to TPNH and oxygen, at least two enzymes and a non-protein cofactor were required for this hydroxylation. The cofactor appeared to be a tetrahydrofolic acid derivative. Kaufmann suggests that it is this cofactor which is directly concerned with the hydroxylation and that the TPNH acts indirectly by producing the tetrahydrofolic acid derivative from an oxidized form. Evidence that a similar mechanism is not involved in steroid 11β-hydroxylation comes from Tomkins, Curran and Michael's observation (1958) that neither folic acid, tetrahydrofolic acid, nor the naturally occurring phenyl alanine hydroxylating cofactor is able to stimulate the 11β-hydroxylase of adrenal mitochondria. It will be most interesting to test the effect of these folic acid derivatives on the 2-hydroxylase described in this thesis.

Another point to be investigated is the possible occurrence of hydroxylation in the 4-position.   Presumably 4-hydroxyoestriol would have a mobility similar to that of the 2-hydroxy isomer in the chromatographic systems used, and small amounts of the 4-hydroxy compound might well have been lost in the purification of the 2-hydroxyoestriol.   This could readily be answered if synthetic 4-hydroxyoestriol was available.

This 2-hydroxylase does not appear to be very specific as it seems able to ortho-hydroxylate oestrone, oestradiol-17β, 17α-ethinyloestradiol-17β and stilboestrol.   In view of the crude nature of the enzyme preparation used in these experiments, this point will need further investigation.

Rat uterus and ovary appear to lack this 2-hydroxylase.   With kidney there may be some doubt even though no 2-hydroxyoestriol could be detected.   This organ can metabolize appreciable amounts of oestriol and it may be that it can produce small amounts of the 2-hydroxy compound but that this is further metabolized to undetected products.   An observation which might

be used to support this suggestion is that rat
kidney homogenates can irreversibly bind
oestradiol-17β to proteins (Reigel and Mueller,
1954). That one should not be too dogmatic in
stating that an organ or tissue does not possess
a certain enzymic activity is exemplified by the
case of 16-oxooestradiol-17β production in liver.
No trace of this compound could be detected by
incubating oestriol with rat liver slices,
homogenates or cell fractions (Section II).
Nevertheless, when nicotinamide was added to a
rat liver microsome plus supernatant preparation,
a small amount of 16-oxooestradiol-17β was
detected.

In all the experiments an appreciable amount
of the added oestriol was apparently lost. The
two main possibilities to account for this are
that the oestriol has been converted to ether-
insoluble material or to ether-soluble, non-
phenolic compounds.

i. Possible Ether-Insoluble Derivatives

Protein binding of the oestriol or one of
its metabolites is a distinct possibility. This
will be discussed later. Another possibility is
the rupture of ring A to form a muconic acid,

e.g.
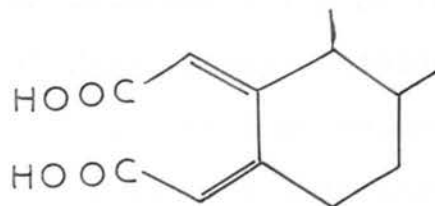
It is conceivable that such a compound would be
ether-soluble at an acid pH, but it will be
discussed here for the sake of convenience.
Man, dog and rabbit can convert benzene to trans-
trans-muconic acid (Williams, 1959).   Ring
rupture would be expected to produce the cis-cis-
isomer, but this does not seem to be the case.
Possible explanations of this are given by
Williams (1959).   He suggests two possible
intermediates in the formation of the muconic
acid.

Benzene

Catechol

trans-trans-
muconic acid
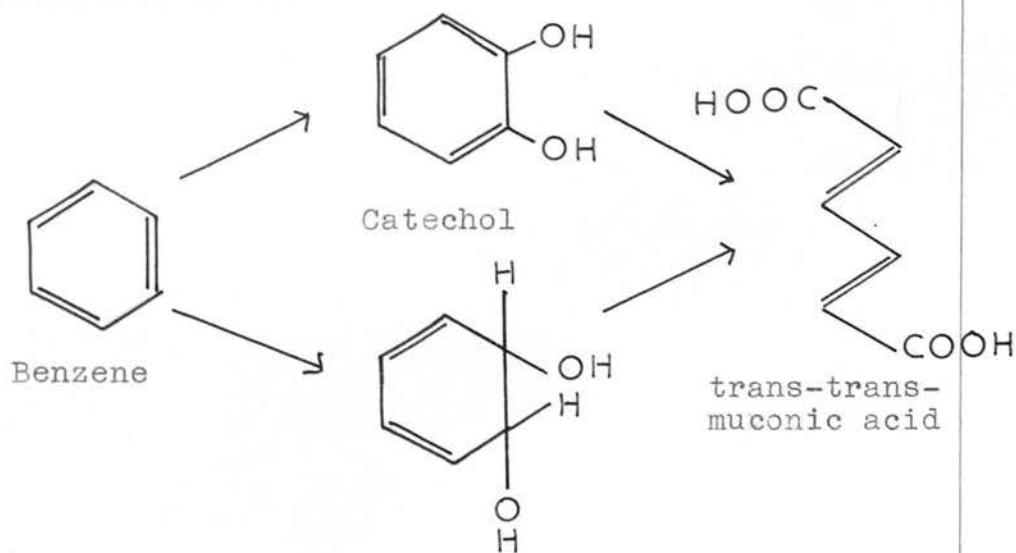
Benzene 1, 2 dihydro 1, 2 diol

In support of catechol being an intermediate is
the fact that Pseudomonas contains a pyro-
catechase which can oxidize catechol to cis-cis-
muconic acid without the intermediate formation
of a quinone (Mason, 1957).

Dr M.M. Coombs (personal communication) has
recently produced evidence that such a reaction
might occur with 2-hydroxyoestriol. On standing
in dilute alkali in air, the benzenoid nature of
the A ring of this compound was completely lost.
The product appeared to be a hydroxy carboxylic
acid.

ii. Ether-Soluble Non-Phenolic Compounds

Quinones can be ruled out as they are Folin
and Ciocalteu positive. A number of polycyclic
aromatic hydrocarbons can be converted in vivo to
partially saturated alcohols (for references see
Williams, 1959).

e.g.

Naphthalene

Naphthalene
1,2 dihydro 1,2 diol

Naphthalene
1,2 dihydro 1 ol
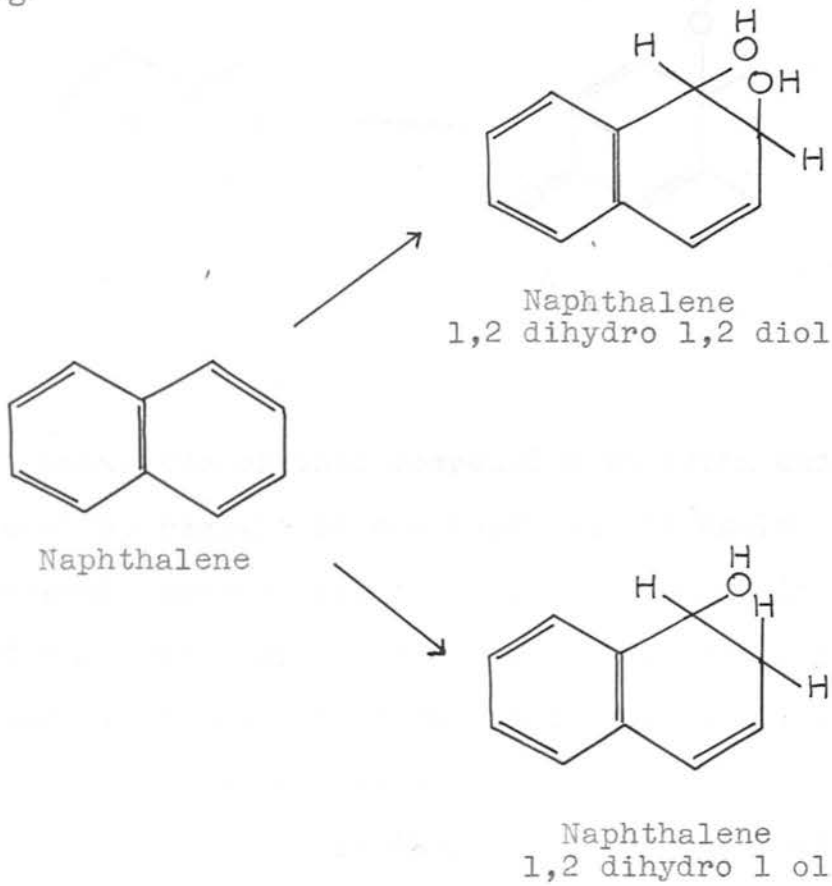
A similar type of reaction might occur with
oestriol.

Mueller, Herranen and Jervel (1958) claim to
have isolated a p-quinol by incubating oestradiol-
17β with rat liver preparations.
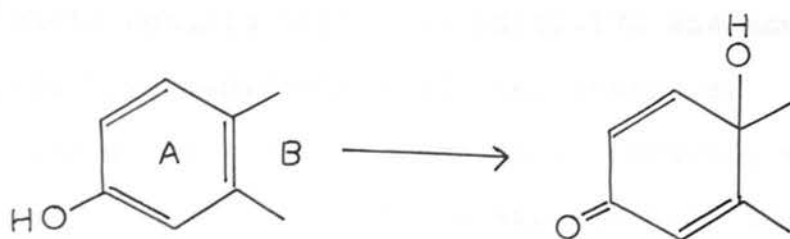
The behaviour of this compound with Folin and Ciocalteu reagent is not known but it would certainly absorb u.v. light very strongly in the 240 mμ region of the spectrum. No such oestriol metabolites could be detected in the experiments described in this thesis.

Rat liver slices (Breuer, Nocke and Knuppen, 1959) and microsomes (Mueller and Rumney, 1957) can 6α-hydroxylate oestradiol-17β, and a similar reaction was anticipated with oestriol. With none of the rat liver preparations described in this thesis could any 6α-hydroxy- or 6-oxooestriol be detected. This was most surprising, especially as the preparation used by Mueller and Rumney was very similar to that used to study the formation of 2-hydroxyoestriol. The most obvious explanation is that the 6α-hydroxylase is a very specific enzyme. In support of this, Breuer, Nocke and Knuppen (1959b) concluded from

their results that oestradiol-17β was more readily 6α-hydroxylated than oestrone. However, because of the large amount of oestriol which is unaccounted for in these experiments, the possibility cannot be ruled out that 6α-hydroxy-oestriol is formed, but that it is further metabolized to undetected products.

The method used throughout these experiments to detect possible metabolites, i.e. Folin and Ciocalteu phenol reagent, means that any 2,3-dimethoxy compounds would not be detected. Axelrod and Tomchick (1958) found that their purified O-methylase would not methylate mono-phenols and would only methylate the 3-hydroxyl group of adrenalin. If, as seems possible, the same enzyme is involved in steroid O-methylation, the formation of such a dimethoxy compound appears unlikely. The possibility that a 2-hydroxy-3-methoxy compound is formed cannot be excluded.
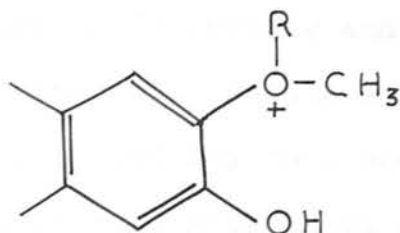
The characteristics of this O-methylation of oestriol bear some similarities to Axelrod's system for the O-methylation of adrenalin (Axelrod and Tomchick, 1958). In view of the apparent lack of specificity of this enzyme, the

O-methylation of 2-hydroxyoestriol may well be
accomplished by the same enzyme.   This would
explain the lower yield of 2-methoxyoestriol when
rabbit slices were used instead of rat liver
slices, as the authors quoted above have shown
that rabbit liver has only one-fiftieth of the
O-methylating activity of an equal weight of rat
liver when adrenalin is used as the substrate.

A number of possible functions can be put
forward for this O-methylation of oestrogens.
The most obvious one is that it is simply due to
the presence of a non-specific O-methylating
enzyme, and thus 2-methoxy oestrogens are bound
to be formed, but that they have no particular
role to play in metabolism.   It is unlikely

An alternative has been suggested by Loke
(1958), namely that the methylation occurs as a
consequence of some biological function of the
corresponding 2-hydroxy compound.   Loke implied
that the methoxy compounds might be the methyl
carriers in a similar way to methionine.   By
analogy with adenosyl methionine, this would
presumably occur via an oxonium ion.

Since the chemical properties of thio-ethers are
not very different from ethers, this suggestion
is not as impossible as would appear at first
sight.   An O-demethylase does exist in rabbit
liver microsomes (Axelrod and Szara, 1958).
This could conceivably be associated with an
'active methionine' function of the O-methyl
compound, but because TPNH, Mg^{2+} and oxygen are
the only essential cofactors, it is unlikely
that 'R' in the above diagram could be the
adenosyl radicle.   This work was carried out
with 3 O-methyl adrenalin.   This, of course,
assumes that the O-demethylation of oestrogens,
if it occurs at all, occurs via a similar
mechanism.

A very interesting biological effect might
be associated with the abnormal metabolism of
these O-methyl compounds.   Schoental (1958) has
pointed out that the O-methyl derivatives of

various poly-nuclear hydrocarbons and azo dyes
are much more carcinogenic in rats and mice than
the parent phenol. Just why this should be is
not clear, but it is well known that alkylating
agents can be potent carcinogens. Could these
O-methyl compounds act in some way as alkylating
agents? If the oxygen-methyl bond could be
activated, this idea would not be so implausible.

That methylation and demethylation might be
involved in carcinogenesis is also suggested by
the work of Conney, Brown, Miller and Miller
(1957). They found that the potent hepatic
carcinogen 4-dimethyl-amino-azo-benzene could be
demethylated by rat and mouse liver preparations
in vitro to give p-amino-azo-benzene. This
compound is much less toxic than the dimethyl
compound. This liver demethylase activity fell
when the rats were fed 3-methyl-4-dimethyl-amino-
azo-benzene and the resulting hepatic tumours
contained very little demethylase. On the other
hand, oral or intraperitoneal administration of
3-methyl-cholanthrene or 3,4-benzpyrene increased
the demethylase activity seven-fold. Like the
O-demethylase discovered by Axelrod and Szara

(1958), this N-demethylase is located in the
microsomes and requires TPNH and oxygen.

It would be most interesting to see if
methoxy oestrogens can be demethylated and, if
so, whether this demethylating and methylating
activity can be influenced by any of these azo
dyes.

Although there is much controversy about the
binding of oestrogens to plasma proteins
(Sandberg, Slaunwhite and Antoniades, 1957) there
is no doubt that oestrogens incubated in vitro
with tissue do become 'protein bound'.   Unless
otherwise stated, the term 'protein bound' will
be used in this discussion to mean irreversibly
bound, i.e. a covalent bond between steroid and
protein.   A reversible type of binding has been
demonstrated with plasma proteins (see Sandberg,
Slaunwhite and Antoniades, 1957), and presumably
a reversible binding must occur during enzymic
metabolism in other tissues.   Steroids could
also associate with tissue lipoproteins.
Investigations into this topic are greatly
hampered by the extreme lability of lipoproteins.
Also the standard method of studying oestrogen

metabolism is to extract the oestrogens with a
lipid solvent such as ether. This might
disrupt any oestrogen-lipoprotein complexes.
This type of binding will not be discussed further.

Szego (1953) and Slaunwhite, Sandberg and
Antoniades (1957) have demonstrated the protein
binding of $[16^{14}C]$ oestrone when this steroid was
incubated with liver slices. The latter workers
showed that this binding decreased if the
incubations were carried out under nitrogen.
Reigel and Mueller (1954) studied this binding in
more detail with the aid of $[16^{14}C]$ oestradiol-17β
and rat liver homogenates. They obtained
maximum binding in the presence of DPN^+, TPN^+ and
an oxidizable substrate. ATP, flavin mono-
nucleotide and flavin adenine dinucleotide did not
influence this binding. The binding was 93%
inhibited when the incubations were carried out
under nitrogen, whereas oxygen stimulated the
binding process. Using a similar system except
that microsomes were used in place of the
homogenate, Mueller and Rumney (1957) detected
five acetone/benzene soluble metabolites of
$[16^{14}C]$ oestradiol-17β. Two of these were
identified as 6α-hydroxyoestradiol-17β and 6-oxo-

oestradiol-17β.   The cofactor requirements for

protein binding are very similar to those for

steroid hydroxylation, and it may be that

hydroxylation plays an integral part in protein

binding.   Reigel and Mueller (1954) suggest that

the binding might occur <u>via</u> the following

sequence of reactions:-

protein
catechol ⟶ o-quinone ⟶ protein bound material
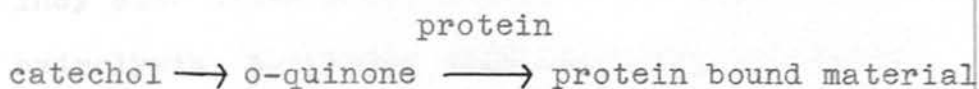
The reaction of quinones with -SH compounds is

well known (Fieser and Fieser, 1950).
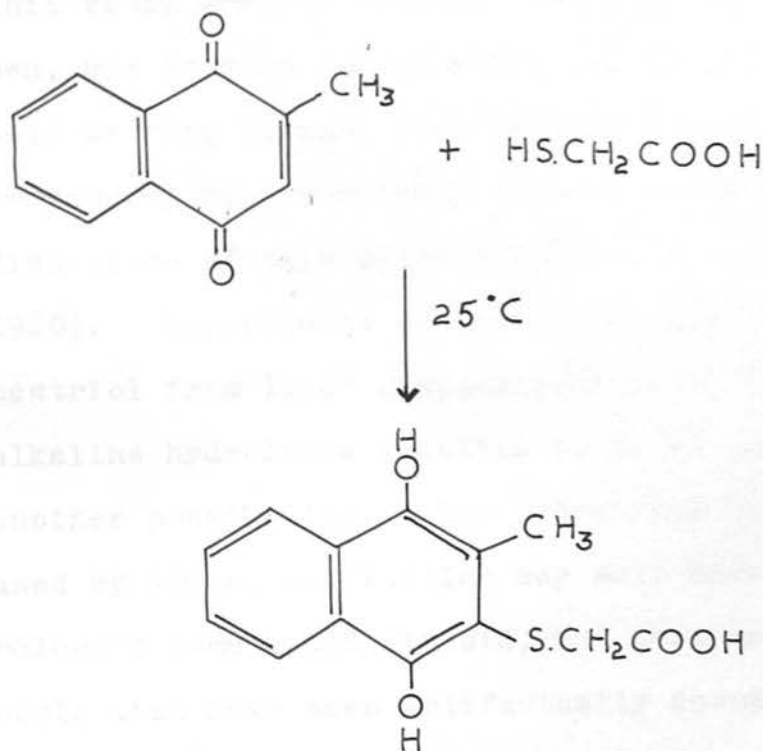
In this respect it is interesting that

naphthalene can combine with glutathione <u>via</u> the

free -SH group of this compound (Booth, Boyland

and Sims, 1960).

Reigel and Mueller (1954) precipitated the 'oestro protein' with cold trichloracetic acid and then extracted the nucleic acids from the protein with hot trichloroacetic acid. The specific activity of the protein did not change, so nucleic acids can be ruled out as the binding material. They also tried acid, alkaline and peptic hydrolysis, performic acid oxidation and Raney nickel oxidation of the 'oestro protein' without releasing any acetone/benzene extractable $\left[{}^{14}C \right]$. This would seem to rule out -SH binding on its own, but it does not eliminate -SH binding as well as ring rupture because such steroids would presumably be appreciably soluble in water (for discussion of this point see Marrian and Sneddon, 1960). Experiments on the extraction of oestriol from liver preparations after acid and alkaline hydrolysis (Section I, 3, b) suggest another possibility. The hydrolysis procedures used by Reigel and Mueller may well have released some bound steroid, but this material could then have been artifactually decomposed by further hydrolysis.

The incubation system used by these authors is so similar to the one used in the experiments

described in this section that some protein
binding of the oestriol or one of its metabolites
must have occurred. The similarity goes further
in that uterus and kidney have only about 10% of
the binding power of liver. This is in agreement
with Szego's results (1953).

Rumney (1958) has studied the effect on the
liver microsomal binding of 1μ g. of $\left[4^{14}C\right]$-
oestradiol-17β of adding other steroids. A 55%
inhibition was produced by 2μ g. of oestriol
which he suggested was due to the oestriol
competing with oestradiol-17β for the binding sites
on the protein. No inhibition was produced by
0.1μ g. of 9α-fluoro-11β-hydroxyprogesterone, but
at the 0.5μ g. level there was a 45-50%
inhibition. Larger amounts of this compound had
very little further effect. This synthetic
steroid might be affecting the enzyme(s) involved
in binding rather than the binding sites on the
protein. It would be interesting to test the
effect of this compound on the metabolism of
oestriol.

The binding of the model compound $\left[8^{14}C\right]$-
tetrahydro-2-naphthol by rat liver microsomal
fraction is paralleled by the formation of a p-
quinol (Hecker and Mueller, 1958) which suggests

that the corresponding oestrogen derivative might
be another possible intermediate in protein
binding.
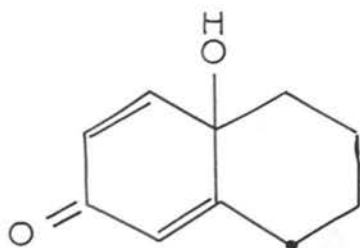
In the same system, oestradiol, 2-fluoro-17-methyl-
oestradiol, 4-fluoro-17-methyloestradiol, 1,17-
dimethyloestradiol and 17-methyloestradiol were
also protein bound.   Whether these fluoro and
methyl compounds are bound in the same way as
oestradiol-17β remains to be seen.   If an o-
dihydroxy or o-quinone structure is involved in
protein binding then it would be interesting to
test the binding of a compound such as 2,4-
difluoro-oestradiol-17β.

     The protein binding of some of the aromatic
hydrocarbons may also help to elucidate the
nature of oestrogen protein binding.   Heidelberger
(1959) has shown that 1:2:5:6-dibenzanthracene is

bound to microsomal and supernatant proteins of
mouse skin by peptide bonds as shown below.
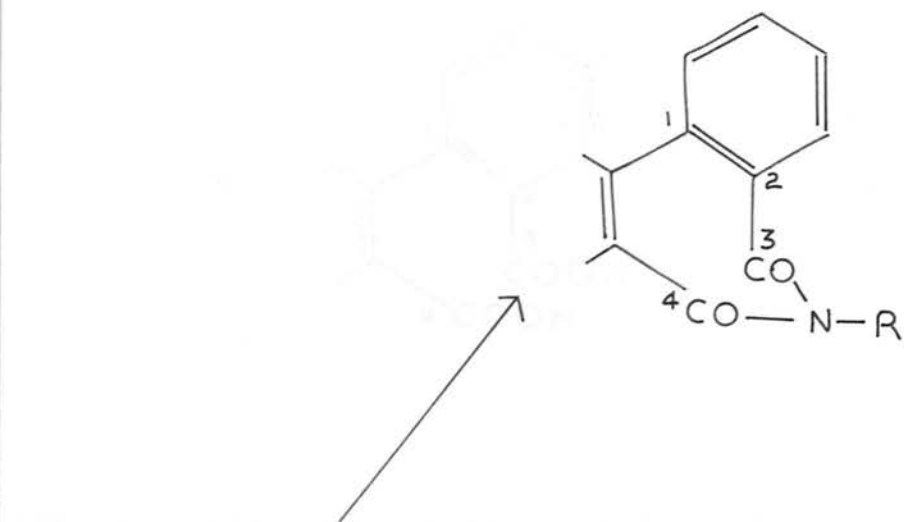
1:2:5:6 Dibenzanthracene
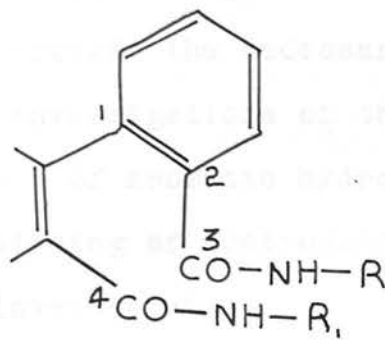
This would suggest the formation of an inter-
mediate muconic acid.
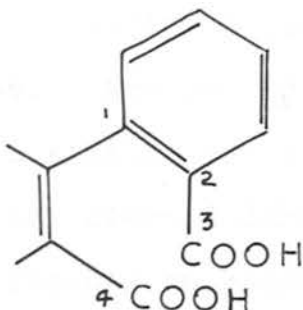
However, this compound is not bound and
Heidelberger suggests that it may be formed _in_
situ. He also found that the 3:4-quinone was
not bound, but the 3-hydroxy compound was.

It is possible that oestrogen binding occurs
via a similar mechanism. If so, it would rule
out the o-quinone suggestion of Reigel and Mueller
(1954). In the near future, synthetic oestrogens
in which the A ring has been opened will be
available, and this should provide the necessary
stimulus for more detailed investigations of this
important topic. The effect of aromatic hydro-
carbons and amines on the binding of oestrogens
would also reward further investigation.

Langer, Alexander and Engel (1959) have put forward evidence that the whole of the 'α-side' of the steroid molecule is important in the union of oestradiol-17β to purified placental oestradiol-17β dehydrogenase.   Marcus and Talalay (1956) have arrived at a similar conclusion for purified bacterial β-hydroxy steroid dehydrogenase.   The preliminary experiments on kidney 16-hydroxy steroid dehydrogenase described in Section II of this thesis could also be explained on this hypothesis.   It may well be that the same type of union is involved in the preliminary stages of protein binding, and hence a complete answer will not be obtained by looking at any one part of the oestrogen molecule.

The next question to be posed is what, if any, is the biological function of these 2-hydroxylated oestrogens?   In view of the manifold metabolic reactions which the liver can perform when given the opportunity, any correlation of function and in vitro metabolic change must be treated with some reserve when this organ is involved.   However, Holmes (1956) has produced evidence that injected oestrone, oestradiol-17β and oestriol are less effective in

stimulating rat uterine water inbibition after
liver damage.   This does not fit in with the
fact that a number of natural and synthetic
oestrogens have direct effects on isolated mouse
vaginal epithelium maintained in tissue culture
(Biggers, 1956).   This apparent disagreement
underlines the need to specify the method by
which the oestrogen effect is measured.   Oestrogens
have been shown to affect a number of tissues
(see Mueller, 1957) in addition to the more
obvious effects on the various organs concerned
with reproduction.   There is no reason to
suppose that there is a common oestrogen effect
to account for this wide diversity of effects,
and consequently the apparent bioactivity of an
oestrogen will depend on the index of activity
used for its assay.   This does in fact appear to
be the case.   Thus oestriol is more active than
oestrone and oestradiol-17β in stimulating
formation of vaginal mucopolysaccharides, and in
stimulating water inbibition by the uterus, but
oestradiol-17β and oestrone are more effective in
increasing uterine dry weight and in promoting
vaginal cornification (Merrill, 1958).   These
points should be borne in mind in the following

discussion.

Dodds (1957) has pointed out that the only feature which is common to all of the natural and synthetic oestrogens is the presence of at least one aromatic nucleus. Hence any modification of the aromatic A ring of the naturally occurring oestrogens should affect their biological potency. In the ensuing discussion it will be assumed that 2-hydroxylation is an activation process, but there is very little evidence for or against this and it may well be a deactivation mechanism. Many of the points raised here are open to further experimentation, and it is hoped that some of these views will be tested in the not too distant future.

It has been known for some time that oestrogens can produce a rapid increase in wet and dry weight of the uterus. Mueller and co-workers have studied this oestrogen-induced uterine growth more fully and have produced good evidence that nucleic acid and protein synthesis are enhanced (Mueller, Herranen and Jervel, 1958). The technique they use is to inject a small dose of a compound such as oestradiol-17β _in vivo_ and after a given time interval, remove the uterus

and study its _in_ _vitro_ biochemistry. However, potent _in_ _vivo_ oestrogens such as stilboestrol, oestrone and oestradiol-17β are inactive when added directly to the _in_ _vitro_ system. This suggests that such compounds must first be 'activated' by some extra-uterine mechanism. In support of this, Mueller (1955) has shown that both 2- and 4-hydroxyoestradiol-17β can stimulate the incorporation of $\left[^{14}C\right]$ formate into uterine proteins when added directly to the _in_ _vitro_ system. In view of the importance of this observation, it is rather surprising that it has not been confirmed or extended to other uterine enzyme systems. If these oestrogen catechols are the 'active oestrogens' in so far as the uterine effect is concerned, the importance of the liver 2-hydroxylase becomes apparent. The effect of oestradiol-17β injection before and after hepatectomy would help to clarify this point.

In 1955, Villee and Gordon detected a DPN-specific _iso_citric dehydrogenase in the supernatant fraction of human placenta which was activated by small amounts of oestradiol-17β. From a kinetic study of this enzyme they concluded that the oestrogen was activating an

inert form of the enzyme. However, a more
detailed investigation of this enzyme by Talalay
and Williams-Ashman (1958) has shown that this
enzyme is in fact TPN-specific, and that Villee's
preparation contained a DPN-TPN transhydrogenase
which was sensitive to trace amounts of oestrone
and oestradiol-17β. They further showed that
this transhydrogenase also possessed oestradiol-
17β dehydrogenase activity, and these two
activities could not be separated by a number of
purification procedures. They put forward the
following attractive suggestion to account for
these findings:-

DPNH ← → OESTRONE ← → TPNH

DPN$^+$ ← → OESTRADIOL-17β ← → TPN$^+$
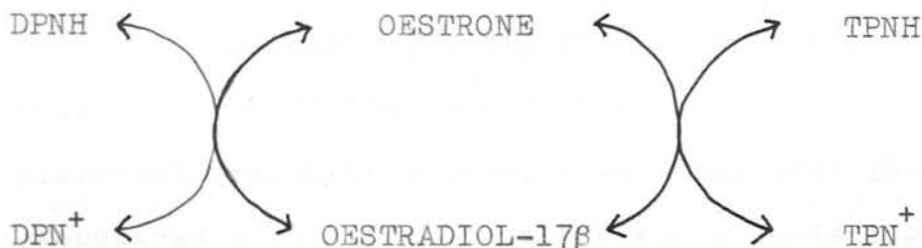
i.e. the transhydrogenase and the oestradiol-17β
dehydrogenase are one and the same enzyme, and
the oestrogen is acting as a cofactor for the
transhydrogenase.

If this mechanism is to account for the biological action of oestrone and oestradiol-17β, it means that these two compounds should be equally bioactive, but this is not the case (Merrill, 1958). Villee has maintained that Talalay's hypothesis does not fit the kinetic data, and he has recently succeeded in separating the dehydrogenase and the transhydrogenase activities (Hagerman and Villee, 1958). As in Mueller's experiments, in vivo oestrogens such as stilboestrol, 17α-ethinyloestradiol-17β and oestriol have no effect on this enzyme preparation. The latter compound may function via 16-oxo-oestradiol-17β (Section I, 9, b). Hollander, Nolan and Hollander (1958), in a comprehensive investigation of the specificity of the placental transhydrogenase, have shown that 16-oxooestradiol-17β does have a small stimulating effect.

It may be that a mechanism based on Talalay's suggestion is functional in another system. Indeed, these two hypotheses could be used to account for the differential effects of the same oestrogen on different tissues. Evidence is accumulating that oestrogens do influence the pyridine nucleotide content of tissues. The oxidation of DPNH by rat uterus

mitochondria is stimulated by oestradiol-17β
(Hollander, Stephens and Adamson, 1959) and
Mongkolkul (personal communication) has shown
that there is an increase in the absolute amounts
of pyridine nucleotides/uterus after the injection
of oestradiol-17β into ovariectomized rats.
Oestradiol-17β can also influence the activity of
a number of hepatic dehydrogenases which could
thus alter the relative concentration of reduced
and oxidized nucleotides (Huggins and Yao, 1959).

A way in which compounds such as stilboestrol,
hexoestrol and 17α-ethinyloestradiol-17β could
affect pyridine nucleotide metabolism is
suggested by Williams-Ashman, Cassman and Klavins
(1959).   They found that potato or mushroom
phenolase, in the presence of trace amounts of a
number of oestrogen derivatives, can stimulate
the oxidation of DPNH and TPNH.   With
oestradiol-17β there is an induction period before
maximal stimulation is obtained, whereas with 4-
hydroxyoestradiol-17β, maximal stimulation was
obtained immediately.   A similar effect was
noted with hexoestrol and 3-hydroxyhexoestrol.
They interpreted these results by the following
reaction sequence:-
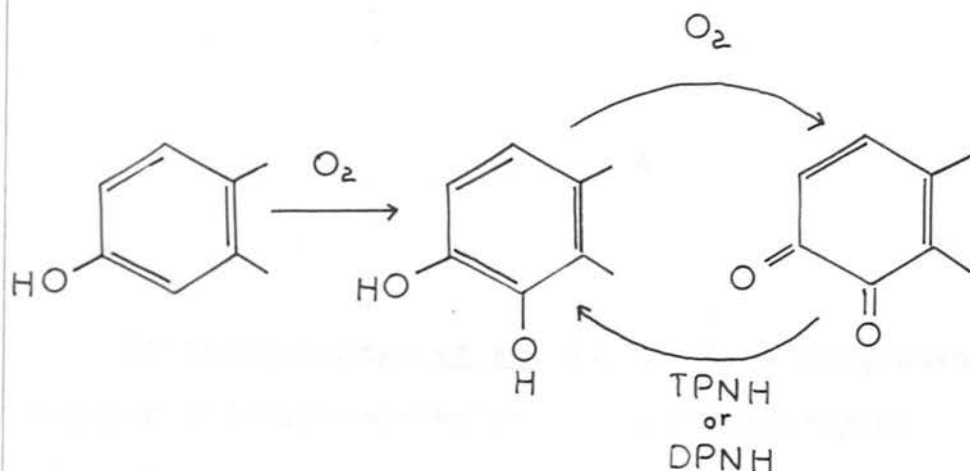
Although these experiments were carried out with
plant enzymes, a similar result could be achieved
by an initial 2-hydroxylation in the liver and an
enzyme in the target organ capable of oxidizing
the catechol to the corresponding quinone.   The
reduction of the quinone back to the catechol
could be non-enzymic.   It is, however, hard to
fit this suggestion in with Dodds' experiments
(1957) with synthetic oestrogens.   He found that
the introduction of any substituent into either
of the benzene rings of stilboestrol or hexoestrol
resulted in a loss of activity.

Williams-Ashman et al. (1959) have suggested
another possible mechanism by which oestrogens
could bring about the oxidation of DPNH based on
a phenol $\rightleftharpoons$ phenoxy radical equilibrium.
They have produced evidence for the following
sequence:-

$$2ROH + O_2 \longrightarrow 2RO\cdot + H_2O_2$$

$$2ROH + H_2O_2 \xrightarrow{\text{peroxidase}} 2RO\cdot + 2H_2O$$

$$4RO\cdot + 2DPNH + 2H^+ \longrightarrow 4ROH + 2DPN^+$$

net
result $\quad 2DPNH + 2H^+ + O_2 \longrightarrow 2DPN^+ + 2H_2O$
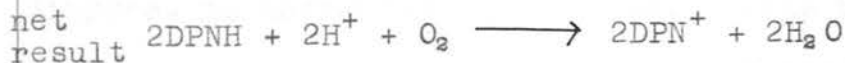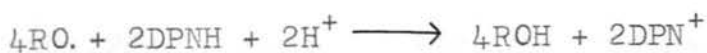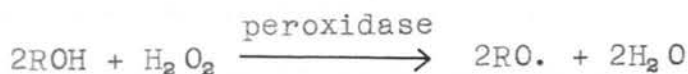
In this respect it is interesting that injection
of physiological doses of natural and synthetic
oestrogens into ovariectomized rats greatly
increases the uterine peroxidase activity (Lucas,
Neufeld, Utterback, Martin and Stotz, 1955).
Again, this sequence is based on experiments with
plant enzymes, but Hollander, Stephens and
Adamson (1959) have put forward evidence to
suggest that a similar mechanism is functional in
rat uterus preparations.

How can this effect of oestrogens on
pyridine nucleotide metabolism be related to
Mueller's experiments on 1-carbon fragment
metabolism and protein synthesis?   Tetrahydro-
folic acid is intimately associated with 1-carbon
fragment metabolism (Huenekens, Osborn and Whitely,
1958) and TPNH is required for the conversion of
dihydro- to tetrahydro-folic acid.   Thus by
increasing the TPNH concentration, the amount of
tetrahydro-folic acid could be increased with a
concomitant stimulation of 'formate' incorporation
into serine and methionine and hence into proteins.
In support of this is the finding of Hertz (1948)
that folic acid is necessary for oestrogen-
stimulated growth of the chicken oviduct.

Another possibility is that by stimulating
DPNH oxidation, ATP synthesis is also increased
and that this provides the necessary energy for
amino acid activation and protein synthesis.   It
is noteworthy that oestradiol-17β can stimulate
the ATP activation of amino acids in the presence
of a rat uterus supernatant enzyme (McCorquadale
and Mueller, 1958).   The demonstration of
Hollander et al. (1959) that certain phenols
stimulate the oxidation of DPNH by rat uterus
particulate fraction is also consistent with the
known site of DPNH oxidation associated with
oxidative phosphorylation.

Quinones have been implicated in oxidative
phosphorylation (Clark, Kirby and Todd, 1958;
Martius, 1959) and it is possible that an
oestrogen quinone derivative may also play a role
in this process.   It would be most interesting to
see if any of these oestrogen o-quinone
derivatives could affect the oxidative
phosphorylation associated with uterus or
mammary gland mitochondria.   Samuels (1958) has
shown that after an injection of oestradiol-17β
into spayed rats, the active uterine phosphorylase

increased two-fold within 48 hr. without affecting the total amount of phosphorylase present. Adenylic acid is known to be involved in the activation of phosphorylase b (Cori and Cori, 1945), and hence any factor affecting adenylic acid could also influence this activation. In view of the complexity of the phosphorylase reaction, this point should not be pressed too far without further experimental work.

In this discussion a great deal of speculation has arisen from two facts, i.e. the demonstration of a 2-hydroxylase and an O-methylase in rat and rabbit liver. The author makes no apology for this and hopes that future research will clarify most of the points made in this discussion.

APPENDIX I

ESTIMATION OF 2-HYDROXYOESTRIOL

AND 2-METHOXYOESTRIOL

1. 2-Hydroxyoestriol

The method described in Section III was
used, the final blue colour being read in a 1 cm.
light path glass cell at 745 mμ. All optical
densities were read against a reagent blank.

2-Hydroxy oestriol (μg.)	1	3	5	7.5	10	21
Optical density (x 10^3)	25	77	132	232	328	698

A series of twelve determinations at the
3 μg. level gave a mean optical density of 0.077
(S.D. ±0.004). A similar series at the 10 μg.
level gave a mean optical density of 0.328
(S.D. ±0.008).

2. 2-Methoxyoestriol

The method described in Section III was
used, the final red colour being read in a 1 cm.
light path glass cell at 522 mμ. All optical
densities were read against a reagent blank.

2-Methoxy-oestriol (µg.)	Optical Density (x 10^3)	2-Methoxy-oestriol (µg.)	Optical Density (x 10^3)
5	88	20	336
7.2	123	25	427
10	167	30	524
15	252	50	912
18.2	305		

A series of twelve determinations at the 7.2 µg. level gave a mean optical density of 0.123 (S.D. ±0.005). A similar series at the 18.2 µg. level gave a mean optical density of 0.305 (S.D. ±0.010).

APPENDIX II

EXPERIMENTAL RESULTS

Section II

1. Effect of varying the incubation time on the conversion of 16α-hydroxyoestrone to oestriol (Fig. 2).

Incubated under Air		Incubated under Nitrogen	
Time hr.	Oestriol Formed %	Time hr.	Oestriol Formed %
0.5	10	0.5	6
1	14	1	18
2	10	2	22
4	6	3.5	16

All incubations contained the following:-
200 mg. of adult male rat liver slices plus 2 ml.
of Krebs-Ringer phosphate, pH 7.4, plus 100 μg.
of 16α-hydroxyoestrone in 0.05 ml. of PG.
Incubated at 37°C.

2. Effect of gas phase on oestriol metabolism
(Fig. 3).

Gas Phase	Oestriol Recovered %
Nitrogen	55
Air	42
Oxygen	13

All incubations contained the following:-

200 mg. of adult male rat liver slices plus 2 ml.

of Krebs-Ringer phosphate, pH 7.4, plus 100 μg.

of oestriol in 0.05 ml. of PG.   Incubated for 1

hr. at 37°C.

Section III

1. Effect of DPN$^+$ and TPN$^+$ on 2-methoxyoestriol and 2-hydroxyoestriol formation by rat liver homogenates (Fig. 8a).

Additions	Oestriol Recovered	2-Methoxy-oestriol Formed	2-Hydroxy-oestriol Formed	Total Recovery
	%	%	%	%
None	63	0	1	64
0.74 μM DPN$^+$ + 4.5 μM sodium lactate	51	4	1	56
1.48 μM DPN$^+$ + 9.0 μM sodium lactate	42	5	1	48
0.67 μM TPN$^+$ + 4.5 μM G-6-P	37	9	5	51
1.34 μM TPN$^+$ + 9.0 μM G-6-P	7	11	6	24
0.67 μM TPN$^+$ + 4.5 μM G-6-P + 0.74 μM DPN$^+$ + 4.5 μM sodium lactate	17	18	6	41
0.74 μM DPN$^+$ + 0.67 μM TPN$^+$	19	15	5	39
0.74 μM DPN$^+$ + 0.67 μM TPN$^+$. Oestriol added just prior to extraction.	73	0	0	73

All incubations contained the following:-

2. Effect of Mg^{2+}, Ca^{2+}, ATP and L-methionine on 1 ml. of 20% homogenate in 0.25 M-sucrose plus 2-methoxyoestriol and 2-hydroxyoestriol formation 1.95 µM of oestriol in 0.035 ml. of PG plus 5 µM by rat liver homogenates (Fig. 26). of ATP plus 60 µM of L-methionine plus 150 µM of $MgCl_2$ plus 240 µM of KCl plus 192 µM of KH_2PO_4, pH 7.4. Final volume 3 ml. Incubated 1 hr. under oxygen, 37°C.

	Recovered oestriol %	oestriol %	oestriol %	Total Recovery %
150 µM $MgCl_2$	20	0	16	36
150 µM $MgCl_2$ + 60 µM L-methionine	25	6	8	39
150 µM $MgCl_2$ + 5 µM ATP	23	6	10	39
150 µM $CaCl_2$ + 5 µM ATP + 60 µM L-methionine	30	0	15	45
150 µM $MgCl_2$ + 5 µM ATP + 60 µM L-methionine	49	13	5	59
150 µM $MgCl_2$ + 5 µM ATP + 60 µM L-methionine. Oestriol added just prior to extraction.	73	0	0	73

All incubations contained the following:-
1 ml. of 20% homogenate in 0.25 M-sucrose plus 0.74 µM TPN^+ plus 0.67 µM of TPN^+ plus 1.95 µM of oestriol in 0.035 ml. of PG plus 192 µM of KH_2PO_4, pH 7.4, plus 240 µM of KCl. Final volume 3 ml. Incubated 1 hr. under oxygen, 37°C.

2. Effect of Mg^{2+}, Ca^{2+}, ATP and L-methionine on
2-methoxyoestriol and 2-hydroxyoestriol formation
by rat liver homogenates (Fig. 8b).

	Oestriol Recovered %	2-Methoxy-oestriol Formed %	2-Hydroxy-oestriol Formed %	Total Recovery %
150 μM MgCl₂	20	0	16	36
150 μM MgCl₂ + 60 μM L-methionine	25	6	8	39
150 μM MgCl₂ + 5 μM ATP	23	0	16	39
150 μM CaCl₂ + 5 μM ATP + 60 μM L-methionine	30	0	15	45
150 μM MgCl₂ + 5μM ATP + 60 μM L-methionine	19	15	5	39
150 μM MgCl₂ + 5 μM ATP + 60 μM L-methionine. Oestriol added just prior to extraction.	73	0	0	73

All incubations contained the following:-

1 ml. of 20% homogenate in 0.25 M-sucrose plus
0.74 μM DPN⁺ plus 0.67 μM of TPN⁺ plus 1.95 μM
of oestriol in 0.035 ml. of PG plus 192 μM of
KH₂PO₄, pH 7.4, plus 240 μM of KCl.   Final
volume 3 ml.   Incubated 1 hr. under oxygen, 37°C.

3. Oestriol metabolism by rat liver microsome plus supernatant fraction.   Effect of DPN$^+$ and TPN$^+$ (Fig. 9a).

Additions	Oestriol Recovered %	2-Hydroxy-oestriol Formed %	Total Recovery %
Extraction control. Oestriol added just prior to extraction.	75	–	75
None	55	1	56
0.74 μM DPN$^+$ + 4.5 μM Na lactate	56	5	61
1.48 μM DPN$^+$ + 9 μM Na lactate	36	8	44
0.67 μM TPN$^+$ + 4.5 μM G-6-P	56	4	60
1.34 μM TPN$^+$ + 9 μM G-6-P	32	13	45
0.74 μM DPN$^+$ + 0.67 μM TPN$^+$ + 4.5 μM Na lactate + 4.5 μM G-6-P	32	16	48
0.74 μM DPN$^+$ + 0.67 μM TPN$^+$ + 4.5 μM Na lactate + 4.5 μM G-6-P + 72 μM nicotinamide	7	15	22

All incubations contained 1 ml. of microsome plus supernatant fraction from a 20% female rat liver homogenate in 0.25 M-sucrose plus 192 μM of potassium phosphate, pH 7.4, plus 1.95 μM of oestriol in 0.025 ml. of PG.   Final volume 3 ml. Incubated 1 hr. under oxygen, 37°C.

4. Effect of preincubating the microsome plus supernatant fraction on the conversion of oestriol to 2-hydroxyoestriol (Fig. 9b).

Additions	Oestriol Recovered %	2-Hydroxy-oestriol Formed %	Total Recovery %
1 ml. microsome + supernatant fraction. Oestriol added just prior to extraction.	74	–	74
1 ml. microsome + supernatant fraction + 4.5 µM Na lactate + 4.5 µM G-6-P	43	13	56
1 ml. preincubated microsome + supernatant fraction + 4.5 µM Na lactate + 4.5 µM G-6-P	49	13	62
1 ml. preincubated microsome + supernatant fraction	53	8	61

All incubations contained 0.74 µM of DPN[+] plus 0.67 µM of TPN[+] plus 192 µM of potassium phosphate, pH 7.4, plus 1.95 µM of oestriol in 0.05 ml. of PG. Final volume 3 ml. Incubated 1 hr. under oxygen, 37°C.

5. Metabolism of oestriol by male rat liver cell

fractions (Section III, 16, a).

Additions	Oestriol Recovered %
Homogenate minus debris and nuclei (≡ 100 mg. wet weight whole liver)	3
Mitochondria (≡ 340 mg. wet weight whole liver)	69
Microsomes (≡ 340 mg. wet weight whole liver)	29
Supernatant (≡ 100 mg. wet weight whole liver)	60
Boiled microsomes	78
Microsomes. Oestriol added just prior to extraction	79

All incubations contained the following:- 1 ml.
of cell fraction suspended in 0.25 M-sucrose plus
0.12 M-nicotinamide plus 0.2 ml. of 0.04 M-ATP
plus 0.2 ml. of 0.045 M-glucose-6-phosphate plus
0.05 ml. of glucose-6-phosphate dehydrogenase
(Sigma Chemical Co. preparation) plus 0.5 mg. of
TPN^+ plus 0.33 ml. of 0.048 M-magnesium sulphate
plus 0.33 ml. of 0.192 M-potassium phosphate,
pH 7.4, plus 0.33 ml. of 0.5 M-potassium
chloride plus 100 μg. of oestriol in 0.05 ml. of
PG.   Incubated 2 hr. in air at 37°C.

APPENDIX III

PREPARATION OF MATERIALS

1. SOLVENTS

All solvents, except ether, were purified by
the methods described in Techniques of Organic
Chemistry, vol. VII, 1955, 2nd edition, edited by
Weissberger, A., Proskauer, E.S., Riddick, J.A.
and Toops, E.E.   A.R. ether was distilled once
just before use.

2. CELITE

Celite 535 (Johns Manville & Co., Ltd.,
London) was purified by partially calcining at
400° for 4 hr., leaving it in 10 N-HCl overnight,
washing with water until free of Cl⁻ and drying in
an oven at 120°C.

3. CHEMICALS

Most of the chemicals used were A.R. quality.

4. STEROIDS

Only those which were kindly supplied by
donors outside this Department will be mentioned.
They are:-

    a. 2-Methoxyoestrone from Dr T.F. Gallagher.

    b. 2-Hydroxyoestradiol-17β from Dr G.C.
       Mueller.

    c. 2-Hydroxyoestriol from Dr M.M. Coombs.
       This was synthesized by a modification
       of the method of Fishman (1958) for the
       synthesis of 2-methoxyoestrogens.

ACKNOWLEDGMENTS

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