A Thesis presented for the Degree
of
Doctor of Philosophy
by
ROGER J.B. KING
Department of Biochemistry
University of Edinburgh

May 1960
Contents

SECTION I

1. GENERAL INTRODUCTION 1

2. EXPERIMENTAL METHODS
   a. Preparation of Tissue Slices 6
   b. Preparation of Homogenate 6
   c. Tissue Fractions 7
   d. Addition of Steroids 8
   e. Preparation of Buffers 8
   f. Gassing of Solutions and Incubation Flasks 8
   g. Kober Reaction 9
   h. Partition Column Chromatography 10
   i. Paper Chromatography 11
   j. Location of Oestrogens on Chromatograms 12
   k. Girard Reaction 12
   l. Sodium Borohydride Reduction 14
   m. Fast Black Salt K Formation 14
   n. Melting Point Determinations 15
   o. Experimental Results 15

SECTION II - IN VITRO METABOLISM OF 16α-HYDROXYOESTRONE, 16-0X0-OESTRADIOL-17β AND 16-0X0-OESTRONE

1. INTRODUCTION 16

2. EXPERIMENTAL METHODS 20

3. DEVELOPMENT OF A METHOD FOR THE EXTRACTION OF OESTRIOL FROM TISSUE INCUBATIONS
   a. Preliminary Experiments 20
   b. Experiments on a Hydrolysis Step to Hydrolyse any Conjugates 22
   c. Final Extraction Procedure 24
   d. Separation and Estimation of Oestriol and 16-epiOestriol 25
4. METABOLISM OF 16α-HYDROXYOESTRONE BY RAT LIVER SLICES
   a. Preliminary Experiments
   b. Effect of Varying the Incubation Time
   c. Effect of Varying the Gas Phase
   d. Effect of Varying the Amount of Tissue
   e. Characterization of the Metabolically Formed Oestriol

5. EXPERIMENTS WITH 16-OXOESTRADIOL-17β AND 16-OXOESTRONE

6. WORKING UP OF 'ELUATE FRACTION' FROM THE LARGE SCALE OESTRIOL METABOLISM EXPERIMENTS (SECTION III, 4)

7. ATTEMPTED DETECTION OF 16-EPIOESTRIOl IN THE MOTHER LIQUORS FROM THE ISOLATION OF 2-METHOXYOESTRIOL (SECTION III, 6)
   a. Preliminary
   b. Acetonide Formation

8. CONVERSION OF OESTRIOL TO 16-OXOESTRADIOL-17β BY RAT LIVER MICROSOME PLUS SUPERNATANT FRACTION

9. METABOLISM OF OESTRIOL BY RAT KIDNEY HOMOGENATES
   a. Preliminary Experiments
   b. Characterization of these Metabolites
      i. Girard Separation
      ii. 16-eipoestriol Fraction
      iii. 16-OXOESTRADIOL-17β Fraction
   c. Effect of DPN+ and TPN+

10. METABOLISM OF 16-EPIOESTRIOl AND 17-EPIOESTRIOl BY RAT KIDNEY HOMOGENATES
    a. 16-eipoestriol
    b. 17-eipoestriol

11. DISCUSSION
SECTION III - OXIDATIVE METABOLISM
OF OESTRIOL

1. INTRODUCTION 60
2. EXPERIMENTAL METHODS 64
   a. Preparation of Homogenates 64
   b. Absorption Spectra 64
3. PRELIMINARY EXPERIMENTS 65
4. LARGE SCALE ISOLATION OF OMI AND OMII 68
5. PRELIMINARY IDENTIFICATION OF OMI 70
   a. Purification of Crude OMI 70
   b. Carbon and Hydrogen Analysis 70
   c. Ultraviolet Absorption Spectrum 71
6. ISOLATION OF CRYSSTALLINE OMI AND
   ITS IDENTIFICATION AS 2-METHOXYOEETRIOL 72
   a. Charcoal Treatment 72
   b. Alumina Chromatography 72
   c. Crystallization and Melting
      Point Determinations 74
   d. Optical Rotation 74
   e. Infrared Spectrum 75
   f. Ultraviolet Absorption Spectrum 75
   g. Kober Reaction 75
7. PRELIMINARY IDENTIFICATION OF RAT OMII 76
   a. Purification of Crude OMII 76
   b. Melting Point Determination 76
   c. Ultraviolet Absorption Spectrum 77
8. STABILITY OF OMII TO A NUMBER OF
   EXPERIMENTAL PROCEDURES 77
   a. Repeated Evaporation of a Methanolic
      Solution of OMII in Air and Nitrogen 77
   b. Treatment with Alkali and Acid 78
   c. Alumina Chromatography 78
   d. Partition Chromatography 79
9. FORMATION OF OMII BY OTHER SPECIES

10. ISOLATION AND PRELIMINARY IDENTIFICATION OF RABBIT OMII
   a. Isolation
   b. Reaction of OMII with Ethylene Diamine
   c. Kober Reaction
   d. Evidence from Co-Factor Requirements for 2-Methoxyoestriol Formation
   e. Ultraviolet Absorption Spectrum in Neutral, Alkaline and Acid Ethanol
   f. Infrared Spectrum

11. ISOLATION OF CRYSTALLINE OMII AND ITS IDENTIFICATION AS 2-HYDROXYOESTRIOL
   a. Preliminary Tests
   b. Crystallization and Melting Point Determination
   c. Infrared Spectrum

12. DEVELOPMENT OF A METHOD FOR THE EXTRACTION, SEPARATION AND ESTIMATION OF 2-METHOXYOESTRIOL, OESTRIOL AND 2-HYDROXYOESTRIOL
   a. Extraction Procedure
   b. Separation of the Three Compounds
      i. 2-Hydroxyoestriol
      ii. 2-Methoxyoestriol and Oestriol
   c. Estimation
      i. Oestriol
      ii. 2-Methoxyoestriol
      iii. 2-Hydroxyoestriol

13. OESTRIOL METABOLISM BY RAT LIVER SLICES

14. OESTRIOL METABOLISM BY RAT LIVER HOMOGENATES. COFACTOR REQUIREMENTS FOR 2-METHOXYOESTRIOL FORMATION
   a. Preliminary Experiments
      i. Choice of Buffer System
      ii. Choice of Cell Fraction
      iii. Optimal Incubation Time
      iv. Final Incubation Conditions
b. Effect of Adding DPN+ and TPN+

c. Effect of Ca2+, Mg2+, ATP
and L-Methionine

d. Preincubation of the Homogenate

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

15. THE METHOXYLATION OF OESTRONE,
OESTRADIOL-17β, 17α-ETHINOESTRADIOL-
17β AND STILBOESTROL

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

a. Preliminary Experiments
i. Choice of a Suitable
Buffer System

ii. Extraction and Separation
of Steroids

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

iv. Choice of Cell Fraction

v. Effect of Adding
Oxidizable Substrates

vi. Final Incubation Conditions

b. DPN+ and TPN+ Requirements

c. Effect of Preincubating the
Microsome plus Supernatant Fraction

d. Effect of Adding Nicotinamide to
the Incubation Medium

e. Effect of Varying the Incubation
Time

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

a. Detection of Two New Metabolites
i. Non-Ketonic Fraction

ii. Ketonic Fraction
b. Preliminary Identification of OMIV
   i. Fast Black Salt K Formation 125
   ii. Sodium Borohydride Reduction 125
   c. Possible Structure of OMIII 125

18. DISCUSSION 126

APPENDIX I  ESTIMATION OF 2-HYDROXYOESTRIOL AND 2-METHYOESTRIOL 158

APPENDIX II  EXPERIMENTAL RESULTS 160

APPENDIX III  PREPARATION OF MATERIALS 168

ACKNOWLEDGMENTS 170

REFERENCES 171
NUMBERING OF THE CARBON ATOMS OF OESTRANE.
SECTION I

Although estrogen (Bulman, 1929; Dey, Veliz and Meyer, 1929), estriol (Marrisa, 1930; estriol-17β (Wintersteiner, Schweik and Milman, 1933) have been isolated from biological material for some time, their metabolic interrelationships have not been fully worked out recently.

From their relative biological potencies, it appeared that estriol-17β is the active primary hormone produced by the ovary and this view is readily supported by evidence. Estriol was thought to be a 

\[
\text{C}_4 \text{H}_7 \text{O} \rightarrow \text{C}_4 \text{H}_7 \text{O} \]

The liver is involved in this deactivation process (see Mayer, 1952).

\[
\text{Oestriol} \rightarrow \text{Oestriol-17β}
\]
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 biological material for some time, their metabolic interrelationships have not been fully studied until recently.

From their relative biological potencies the suggestion was put forward that oestradiol-17β is the active primary hormone produced by the ovaries and that this is readily converted to oestrone. Oestriol was thought to be a deactivated metabolite of one or both of these. The liver is involved in this deactivation process (see Mayer, 1952).

\[
\begin{align*}
\text{Oestrone} & \xrightarrow{\text{C}} \text{Oestradiol-17β} \\
\text{Oestradiol-17β} & \xrightarrow{\text{D}} \text{Oestriol}
\end{align*}
\]
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 $[16^{14}\text{C}]$ oestradiol-17β or $[16^{14}\text{C}]$ oestrone injected into women gives rise to urinary $[1^{14}\text{C}]$ 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 $[1^{14}\text{C}]$ oestrone or $[1^{14}\text{C}]$ oestradiol-17β in the urine after injection of $[16^{14}\text{C}]$ oestriol into women. With the isolation of 16-oxooestrone (Serchi, 1953), 16-oxooestradiol-17β (Levitz, Spitzer and Twombly, 1956; Layne and Marrian, 1959), 16α-hydroxyoestrone (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 \([16\, ^{14}C]oestrone\) into women and measured the \(^{14}C/tritium\) ratios in the urinary oestrone, oestradiol-17β, oestriol, 16-epioestriol 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 \([^{14}C]acetate\). Thus Rabinowitz and Dowben (1955) found that dog ovary slices or homogenates could synthesize \([^{14}C]oestradiol-17β\) and \([^{14}C]oestrone\) from \([\, 2^{14}C]acetate\). In all the experiments they performed, the specific activity of the oestradiol-17β was greater than that of the oestrone. On the precursor-product relationship 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:

\[ \text{DPNH} \rightleftharpoons OESTRONE \rightleftharpoons \text{TPNH} \]
\[ \text{DPN}^+ \rightleftharpoons \text{OESTRADIOL-17β} \rightleftharpoons \text{TPN}^+ \]
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 transhydrogenase. 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 m$\mu$, 512.5 m$\mu$ and 545 m$\mu$. By applying the Allen correction (Allen, 1950), the corrected optical density at 512.5 m$\mu$ could be obtained.
This is achieved by subtracting the average of the readings at 480 m\(\mu\) and 545 m\(\mu\) from that at 512.5 m\(\mu\). The absorption maximum of the typical Kober colour is 512.5 m\(\mu\).

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-epioestriol and 17-epi-oestriol are eluted with 2→8 ml. of ethylene dichloride, and oestriol with 10→35 ml. of this solvent.

i. Paper Chromatography

This was carried out in a thermostatically controlled room at 20°±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, 1951). 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 μg. of the oestrogen in 0.1 ml. of methanol, 0.1 ml. of 20% Na₂CO₃ 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. **Melting Point Determinations**

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

o. **Experimental Results**

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

SECTION II

IN VITRO METABOLISM OF 16α-HYDROXYEstrONE,

16-OXOEstradiol-17β AND 16-OXOEstrONE

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

In 1957, Johnson and Crisi put forward the following suggestion, based on the relative liposolubilities of a number of compounds which they had tested.
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.

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.
They produced evidence for such a pathway when, after injection of 16-oxoestrone 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-oxoestrone 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.

\[
\begin{align*}
\text{Oestrone} & \rightarrow \text{16α-Hydroxyoestrone} & \rightarrow \text{Oestriol}
\end{align*}
\]
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 undergoes 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-oxoestradiol-17β and 16-oxoestriol.

Levitz, Spitzer and Twombly (1958) have shown that [16\(^{14}\)C] oestriol can be converted to 16-oxoestradiol-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 EXTRAC TION 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-oxoestradiol-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-oxoestradiol-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 $[16\text{H}^{14}\text{C}]$-oestrone and separated the slices and medium, only 25% of the $[14\text{C}]$ 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 $[8_{14}C]$ tetrahydro-2-naphthol.

\[
\text{HO}\quad \text{HO}
\]

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) NaHCO₃.

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-H₂SO₄ 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 16α-OH OESTRONE METABOLITES FROM CELITE COLUMN WITH THE SYSTEM 70 METHANOL:30 WATER/ETHYLENE DICHLORIDE.
<table>
<thead>
<tr>
<th>Steroid Added</th>
<th>Oestriol Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>100 µg. oestriol in 0.05 ml. PG added after the incubation</td>
<td>69</td>
</tr>
<tr>
<td>100 µg. oestriol in 0.05 ml. PG added before the incubation</td>
<td>32</td>
</tr>
<tr>
<td>100 µg. 16α-hydroxyoestrone in 0.05 ml. PG added before the incubation</td>
<td>14</td>
</tr>
</tbody>
</table>

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 to 30 ml. of ethylene dichloride had the same elution pattern as
FIG. 2. CONVERSION OF 16α-OH OESTRONE TO OESTRIOL BY RAT LIVER SLICES.

200 mg. RAT LIVER SLICES + 2 ml. KREBS-RINGER PHOSPHATE pH 7.4 + 100 μg. 16α-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→8 ml. is of some interest. The most likely second metabolite of 16α-hydroxyoestrone is 17-epi-oestriol. 17-epi-oestriol 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-epi-oestriol. There is no trace of any α-ketols or 16-epi-oestriol. Assuming that 17-epi-oestriol gives the same intensity as oestriol in the Kober reaction, then there is about 1% conversion of 16α-hydroxyoestrone to 17-epi-oestriol.

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.

200 mg. RAT LIVER SLICES + 2 ml. KREBS-RINGER PHOSPHATE pH 7.4 + 100 μg. OESTRIOL IN 0.05 ml. 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.

<table>
<thead>
<tr>
<th>Wet weight of tissue mg.</th>
<th>Conversion to oestriol %</th>
<th>Wet weight of tissue mg.</th>
<th>Conversion to oestriol %</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>20</td>
<td>300</td>
<td>25</td>
</tr>
<tr>
<td>200</td>
<td>23</td>
<td>400</td>
<td>14</td>
</tr>
</tbody>
</table>

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−H₂SO₄, and extracted three times with 100 ml. of ether. The ether extract was washed once with 50 ml. of 5% (w/v) NaHCO₃ 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-hydroxyoestrone 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.
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.

<table>
<thead>
<tr>
<th>Steroid Incubated</th>
<th>Oestriol Formed</th>
<th>16-epioestriol Formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>16-Oxooestradiol-17β</td>
<td>2</td>
<td>17</td>
</tr>
<tr>
<td>16-Oxoestrone</td>
<td>6</td>
<td>16</td>
</tr>
</tbody>
</table>
6. WORKING UP OF 'ELUATE FRACTION' FROM THE LARGE SCALE OESTRIOL METABOLISM EXPERIMENTS (Section III, 4)

If any compounds less polar than 17-epi-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-methoxyoestradiol.
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.
ether was evaporated to dryness to give the hydrolysed acetonide fraction.

Portions (30 µg.) of both the hydrolysed and unhydrolysed acetonide fraction were chromatographed 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-methoxyoestriol 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-epioestriol or to any of the α-ketols under these incubation conditions.

8. CONVERSION OF OESTRIOL TO 16-OXO-OESTRADIOL-17β BY RAT LIVER MICROSONE PLUS SUPERNATANT FRACTION

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-oxoestradiol-17β in the formamide/chloroform system. It was ketonic.

On reducing this compound with sodium borohydride it was converted mainly to 16-epi-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/chloroform 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-epioestriol 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(γC = 0 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-oxoestradiol-17β.

c. Effect of DPN+ and TPN+

The following duplicate incubations were carried out:

<table>
<thead>
<tr>
<th>Incubation Tube</th>
<th>Additions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100 µg. oestriol in 0.025 ml. PG added just prior to extraction</td>
</tr>
<tr>
<td>2</td>
<td>100 µg. oestriol in 0.025 ml. PG added just before incubation</td>
</tr>
<tr>
<td>3</td>
<td>100 µg. oestriol in 0.025 ml. PG added just before incubation + ( \frac{1}{2} ) mg. DPN+</td>
</tr>
<tr>
<td>4</td>
<td>100 µg. oestriol in 0.025 ml. PG added just before incubation + ( \frac{1}{2} ) mg. TPN+</td>
</tr>
<tr>
<td>5</td>
<td>100 µg. oestriol in 0.025 ml. PG added just before incubation + ( \frac{1}{2} ) mg. DPN+ + ( \frac{1}{2} ) mg. TPN+</td>
</tr>
</tbody>
</table>

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 borohydride 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 Pangelo, 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.

<table>
<thead>
<tr>
<th>Steroid Incubated</th>
<th>Metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oestriol</td>
<td>15-20% 16-oxoestradiol-17β; 1% 16-epioestriol</td>
</tr>
<tr>
<td>16-epiOestriol</td>
<td>5% 16-oxoestradiol-17β; 1% oestriol</td>
</tr>
<tr>
<td>17-epiOestriol</td>
<td>15% 16-oxoestradiol-17α; 1% 16:17-epioestriol</td>
</tr>
</tbody>
</table>

16:17-epiOestriol was not tested, but presumably would give 16-oxoestradiol-17α and 17-epioestriol.
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 can be converted in small yield into 16α-hydroxy- and 16β-hydroxyoestrone and 16-oxoestradiol-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 [16\(^{14}\)C]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-epi-oestriol. 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-epi-oestriol

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

16-epi-Oestriol 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 \([16^{14}C]\) 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:

\[
\begin{array}{c}
\text{16β-Hydroxyoestrone} \\
\text{16α-Hydroxyoestrone} \\
\text{16-Oxooestradiol-17β} \\
\text{Oestriol} \\
\text{16-epiOestriol}
\end{array}
\]
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 pathway 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-oxooestradiol-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-Hydroxyoestrone

16-epiOestriol

16-Oxo-oestrone

Oestriol

16α-Hydroxyoestrone

17-epiOestriol
Purified human placental oestradiol-17\(\beta\) dehydrogenase can reduce 16-oxoestrone to 16-oxoestradiol-17\(\beta\) (Langer, Alexander and Engel, 1959).

Thus these \textit{in vitro} studies indicate that the main metabolite of 16\(\alpha\)-hydroxyoestrone is oestriol, whilst 16-oxoestrone and 16-oxoestradiol-17\(\beta\) 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 \textit{in vivo} route for oestriol synthesis in the liver is via 16\(\alpha\)-hydroxyoestrone. So far, no '\textit{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 16\(\alpha\)-hydroxy- and 16\(\beta\)-hydroxy-intermediates has been suggested for the conversion of dehydroepiandrosterone to androst-5-ene-3\(\beta\):16\(\alpha\):17\(\beta\)-triol and androst-5-ene-3\(\beta\):16\(\beta\):17\(\beta\)-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 [16\(^1\)H\(_2\)]-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.

VI OESTRIOL.
VII 16-EPIOESTRIOL.
VIII 17-EPIOESTRIOL.
IX 16-OXO OESTRADIOL-17δ.
X 16,17-EPIOESTRIOL.

→ MAJOR PATHWAY.

→ HYPOTHETICAL PATHWAY.
FIG. 5. 3-DIMENSIONAL REPRESENTATION OF THE C & D RINGS OF 16β-OH OESTRONE AND 16α-OH OESTRONE.

--- β BOND. --- α BOND.

16α-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-epi oestriol. 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, Rondak, using a bioassay technique demonstrated that rat and guinea-pig liver slices would deesterify oestrone and that this was an oxidative process. He also showed that acid hydrolysis of the incubation medium did not increase the biocactivity 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 (Zingman and Lauer, 1957).

SECTION III

OXIDATIVE METABOLISM OF OESTRIOL

The oxidative metabolism of oestradiol by liver preparations of a number of species has also been demonstrated by numerous workers using both classical chemical and physical estimation and identification methods. Thus Ryan and Engel (1951), using counter-current distribution and fluorescent 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 oestrone was also enhanced. Similarly, Kiel and Musling (1956) have demonstrated that the irreversible protein binding of $^{131}I$ oestradiol-17β by fortified rat liver homogenates is 95%.
1. INTRODUCTION

In 1934 Zondek, using a bioassay technique demonstrated that rat and guinea-pig liver slices could 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 (Dingemanse and Laquer, 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 steroid was also enhanced. Similarly, Reigel and Mueller (1954) have demonstrated that the irreversible protein binding of $[16^{14}C]$oestradiol-17β by fortified rat liver homogenates is 93%
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 $\left[16^{14}C\right]$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 $\left[16^{14}C\right]$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 0-methyl adrenalin (Axelrod and Tomchik, 1958) and other 0-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\text{\textsuperscript{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 $[16^{14}\text{C}]$oestriol, when injected into women is partly excreted as 16-oxoestradiol-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 μg. of oestriol were incubated with rat liver slices under oxygen, only about 12 μg. 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:**

- 100 µg. 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 20\mu 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-17ß (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 formamid/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 Blender. 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 (C₁₉H₂₆O₄) containing 1 molecule of acetone of crystallization (C₃H₆O). This was not satisfactory.
c. **Ultraviolet Absorption Spectrum**

A sample of OMI (118 \( \mu \)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_{\text{max.}} = 288 \text{ m\(\mu\), } \varepsilon = 4600 \]  
\[ \lambda_{\text{min.}} = 253 \text{ m\(\mu\), } \varepsilon = 634 \]  

Fishman and Gallagher (1959) have reported the following values for 2-methoxyoestriol:

\[ \lambda_{\text{max.}} \text{ ethanol} = 286 \text{ m\(\mu\), } \varepsilon = 3700 \]  
\[ \lambda_{\text{min.}} \text{ ethanol} = 253 \text{ m\(\mu\), } \varepsilon = 350 \]  

This provided further evidence that OMI was 2-methoxyoestriol as, apart from 6-oxygenated derivatives, most oestrogens have a \( \lambda_{\text{max.}} \) of 280 m\(\mu\). 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 m\(\mu\) peak shifted to 300 m\(\mu\) (\( \varepsilon = 5266 \)) and a new maximum appeared at 245 m\(\mu\) (\( \varepsilon = 7990 \)). The corresponding minima were at 233 m\(\mu\) (\( \varepsilon = 5344 \)) and 273 m\(\mu\) (\( \varepsilon = 1432 \)). 2-Methoxyoestrone develops a maximum at 245 m\(\mu\) 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 CRYSSTALLINE 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-Methoxyoestriol 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.
- 5.4 μg. 2-METHOXY OESTRIOL.
- 15 μg. 2-METHOXY OESTRIOL HEATED FOR 4 MINS. WITH KOBER REAGENT.
ethanol and the optical rotation measured -
\[ [\alpha]_D^{17.2} = +81.1^\circ \]
2-Methoxyoestriol -
\[ [\alpha]_D^{25} = +83^\circ \text{ (ethanol)} \]
(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 \( \mu \text{g.} \)) was dissolved in 3 ml. of ethanol and its u.v. spectrum measured.

\[
\lambda_{\text{max.}} 287 \text{ m}\mu \quad (\varepsilon = 3980) \quad \lambda_{\text{min.}} 253 \text{ m}\mu
\quad (\varepsilon = 676). \quad \text{Both of these extinction coefficients are high as compared with the figures quoted by Fishman and Gallagher (1959) } \lambda_{\text{ethanol max.}} 286 \text{ m}\mu
\quad (\varepsilon = 3700) \quad \lambda_{\text{min.}} 253 \text{ m}\mu \quad (\varepsilon = 350).\]

g. Kober Reaction

OMI (54 \( \mu \text{g.} \)) 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 m\( \mu \). This is similar
to 2-methoxyestrone (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 m\(\mu\) with a minimum at 254 m\(\mu\). This suggested that it might be related to 2-methoxyestriol.

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 (200 \mu g.) 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 supernatant 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 25 μg. each of OMII and oestriol and 8 μg. each of hydroquinone,
catechol, resorcinol and p-benzoquinone. This gives about the same equivalent weight of each compound.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Colour in air</th>
<th>Colour in u.v. light</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oestriol</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>OMII</td>
<td>yellow</td>
<td>intense yellow</td>
</tr>
<tr>
<td>Catechol</td>
<td>yellow-brown</td>
<td>intense yellow</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>purple-brown</td>
<td>faint brown</td>
</tr>
<tr>
<td>Resorcinol</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>p-Benzquinone</td>
<td>purple</td>
<td>faint brownish-yellow</td>
</tr>
</tbody>
</table>

With catechol, this colour is supposed to be due to the following reaction (Weil-Malherle, 1959):

\[
\text{H}_2\text{O}\text{O}_2\text{OH}^-\rightarrow \text{H}_2\text{N}\text{Hz}\text{O}_2\text{NC}_{\text{H}_2}\text{N}+2\text{NH}_2\text{CH}_2\text{CH}_2\text{NH}_2
\]
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.

![Chemical structure](image)

Adrenalin undergoes a similar type of oxidation in the presence of alkali to produce adrenochrome.

![Chemical structure](image)

Adrenalin Adrenochrome

The experiments described in Section XIII, indicate that DPN⁺ or TPN⁺, Mg²⁺, ATP and L-methionine are needed for the conversion of oestriol to 2-methoxyoestriol by liver homogenates.
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 µg.) 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+, Mg2+, ATP and L-methionine are needed for the conversion of oestriol to 2-methoxyoestriol by liver homogenates.
FIG. 7. ULTRAVIOLET ABSORPTION SPECTRUM OF OMII.

1196 OMII/3m. ETHANOL
1196 OMII/3m. ETHANOL + O.1m. N NaOH.
FOR 30mins, O.15m. N HC1 ADDED.

AFTER STANDING FOR 30mins, O.15m. N HC1 ADDED.

WAVELENGTH

OPTICAL DENSITY.
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 $\mu$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 m$\mu$, $\lambda_{max.}$ 287 m$\mu$. 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 m$\mu$. The spectrum in neutral ethanol is similar to that of 2-hydroxyoestradiol-17$\beta$ ($\lambda_{min.}$ 255 m$\mu$. $\lambda_{max.}$ 288 m$\mu$), but different to that of 4-hydroxyoestradiol-17$\beta$ ($\lambda_{min.}$ 254 m$\mu$. $\lambda_{max.}$ 280 m$\mu$. 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-hydroxyoestriol 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-hydroxyoestriol.
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 chromatographed for about 3\(\frac{1}{2}\) hr. in the 70% acetic acid/ethylene dichloride system. Standard 2-hydroxyoestriol 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-epi-oestriol 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-epi-oestriol. 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-methoxyoestriol from chloroform by N-NaOH (Section II, 7, b).

When this residue was chromatographed on a celite column as described above, the 2-methoxyoestriol 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 µg 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 µg 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 (15 µg.) plus 3.5 ml. of Kober reagent were heated in a boiling water bath for 5 min. No hydroquinone 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 m/ 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 50\( \mu \) 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 m\( \mu \) measured. The amount of 2-methoxyoestriol/tube was then found by reference to a calibration graph. At the 7\( \mu \) g. level, this method gave an estimate to within \( \pm 4\% \) (see Appendix 1).

iii. 2-Hydroxyoestriol. In the preliminary experiments with crude 2-hydroxyoestriol, it appeared to behave in a similar way to 2-methoxyoestriol 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 (5 µg.) 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 µg./3 ml. of final volume. At the 10 µg. level this method gives an estimate to within ±2.54% (see Appendix I).

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 μg. of oestriol in 0.05 ml. of PG. The tubes were incubated under oxygen for 2 hr. at 37°C.

<table>
<thead>
<tr>
<th></th>
<th>Oestriol recovered</th>
<th>2-Methoxy-oestriol formed</th>
<th>2-Hydroxy-oestriol formed</th>
<th>Total recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slices boiled 1 min. prior to incubation</td>
<td>70 %</td>
<td>1 %</td>
<td>0 %</td>
<td>71 %</td>
</tr>
<tr>
<td>Viable slices. Oestriol added just prior to extraction</td>
<td>70 %</td>
<td>0 %</td>
<td>0 %</td>
<td>70 %</td>
</tr>
<tr>
<td>Viable slices</td>
<td>5 %</td>
<td>10 %</td>
<td>18 %</td>
<td>33 %</td>
</tr>
</tbody>
</table>

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 $5 \times 10^3$ 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 methoxylolation 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 $\mu$g. 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 chromatographed 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-hydroxyoestriol.

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 μg. of 2-methoxyoestriol but less of the 2-hydroxy compound than in tube 3. There was also less oestriol.

Consequently the potassium chloride/Mg2+/potassium phosphate buffer was used in this study. The ability of this buffer, but not the Tris/HCl/MgCl2 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.

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>Oestriol Recovered</th>
<th>2-Methoxyoestriol Formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>hr.</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>13</td>
<td>16</td>
</tr>
<tr>
<td>1</td>
<td>13</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>17</td>
</tr>
<tr>
<td>4</td>
<td>14</td>
<td>16</td>
</tr>
</tbody>
</table>

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 150μM MgCl₂ + 5μM. ATP + 60μM. L-METHIONINE.

[Graph showing different metabolites and their conversion percentages for different conditions.]

FIG. 8B. OESTRIOL METABOLISM BY RAT LIVER HOMOGENATES. EFFECT OF MAGNESIUM, ATP & L-METHIONINE.

ALL INCUBATIONS CONTAINED 0.74μM. DPN⁺ + 0.64μM. TPN⁺.

[Graph showing different metabolites and their conversion percentages for different conditions with magnesium, ATP, and L-methionine variations.]
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 μg. of oestriol to the complete incubation medium just prior to extraction. The 2-methoxyoestriol and 2-hydroxyoestriol 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-methoxyoestriol. 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).
<table>
<thead>
<tr>
<th>Additions</th>
<th>Oestriol Recovered %</th>
<th>2-Methoxyoestril %</th>
<th>2-Hydroxyoestril %</th>
<th>Total %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction control</td>
<td>72</td>
<td>-</td>
<td>-</td>
<td>72</td>
</tr>
<tr>
<td>1 ml. homogenate</td>
<td>14</td>
<td>3</td>
<td>14</td>
<td>31</td>
</tr>
<tr>
<td>1 ml. homogenate + 5μM ATP</td>
<td>10</td>
<td>15</td>
<td>8</td>
<td>33</td>
</tr>
<tr>
<td>1 ml. preincubated homogenate</td>
<td>41</td>
<td>0</td>
<td>10</td>
<td>51</td>
</tr>
<tr>
<td>1 ml. preincubated homogenate +</td>
<td>19</td>
<td>0</td>
<td>11</td>
<td>30</td>
</tr>
<tr>
<td>5μM ATP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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-Hydroxyoestril to 2-Methoxyoestril

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-methoxyoestriol. 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-methoxyoestriol was formed.

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

\[
\begin{align*}
\text{DPNH or TPNH} & \quad \rightarrow \quad \text{ATP, Mg}^{2+} \\
oestriol & \quad \rightarrow \quad 2\text{-hydroxyoestriol} & \quad \rightarrow \quad 2\text{-methoxyoestriol} \\
& \quad \rightarrow \quad O_2 & \quad \rightarrow \quad \text{L-methionine}
\end{align*}
\]

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-ETHINYL OESTRADIOL-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 3½ 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-methoxyoestradiol-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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R_F$</th>
<th>Compound</th>
<th>$R_F$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oestradiol-17β</td>
<td>0.27</td>
<td>Oestrone</td>
<td>0.57</td>
</tr>
<tr>
<td>17α-Ethinyl-oestradiol-17β</td>
<td>0.37</td>
<td>'2-Methoxy-17α-ethinyl-oestradiol-17β'</td>
<td>0.60</td>
</tr>
<tr>
<td>Stilboestrol a</td>
<td>0.25</td>
<td>'Methoxy-stilboestrol'</td>
<td>0.60</td>
</tr>
<tr>
<td>Stilboestrol b</td>
<td>0.43</td>
<td>2-Methoxyoestrone</td>
<td>0.73</td>
</tr>
</tbody>
</table>
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 3μg. 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 4.5 hr. No compound more polar than oestriol could be detected in any of the tubes.

As a preliminary experiment, this indicated that oestrone, 17a-ethinyloestadiol-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-HYDROXOEESTRIOL

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 μg. 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.
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Oestriol Recovered</th>
<th>2-Hydroxyoestriol Formed</th>
<th>Total Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female liver (extraction control)</td>
<td>81 %</td>
<td>- %</td>
<td>81 %</td>
</tr>
<tr>
<td>Female liver</td>
<td>25 %</td>
<td>22 %</td>
<td>47 %</td>
</tr>
<tr>
<td>Male liver</td>
<td>35 %</td>
<td>12 %</td>
<td>47 %</td>
</tr>
<tr>
<td>Female kidney</td>
<td>37 %</td>
<td>1 %</td>
<td>38 %</td>
</tr>
<tr>
<td>'Normal' uterus</td>
<td>76 %</td>
<td>1 %</td>
<td>77 %</td>
</tr>
<tr>
<td>Ovary</td>
<td>70%</td>
<td>1%</td>
<td>71%</td>
</tr>
</tbody>
</table>

- only one estimation
- only two estimations

Unfortunately the oestrous 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-oxoestradiol-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:

<table>
<thead>
<tr>
<th>Liver Preparation</th>
<th>Oestriol Recovered</th>
<th>2-Hydroxy-oestriol Formed</th>
<th>Total Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole homogenate. No oestriol added.</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Whole homogenate. Oestriol added</td>
<td>6</td>
<td>30</td>
<td>36</td>
</tr>
<tr>
<td>Homogenate minus cell debris and nuclei</td>
<td>9</td>
<td>30</td>
<td>39</td>
</tr>
<tr>
<td>Microsome plus supernatant fraction</td>
<td>24</td>
<td>17</td>
<td>41</td>
</tr>
</tbody>
</table>

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 supernatant 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, (192 μ 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 (4.5 μ moles) sodium lactate and 0.1 ml. of 0.045 M (4.5 μ 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 MICROSONE + SUPERNATANT FRACTION.

A) EFFECT OF DPN" AND TPN".

B) EFFECT OF PREINCUBATING THE MICROSONE + SUPERNATANT FRACTION.

ALL INCUBATIONS CONTAINED 0.75 mM DPN" + 0.67 mM 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.

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>Oestriol Recovered</th>
<th>2-Hydroxyoestriol Formed</th>
<th>Total Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>hr.</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>82</td>
<td>-</td>
<td>82</td>
</tr>
<tr>
<td>0.5</td>
<td>8</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td>1.5</td>
<td>4</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>14</td>
<td>17</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>9</td>
<td>12</td>
</tr>
</tbody>
</table>

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 6a-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-oxoestradiol-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-oxoestradiol-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-oxoestradiol-17β (Loke, 1958).

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

c. Possible Structure of OMIII

The most likely compound is 2-hydroxy-16-oxoestradiol-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 absorb 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.

1. 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,
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.
In support of catechol being an intermediate is the fact that *Pseudomonas* contains a pyrocatechase 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).
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 m\(\mu\) 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 6\(\alpha\)-hydroxylate oestradiol-17\(\beta\), and a similar reaction was anticipated with oestriol. With none of the rat liver preparations described in this thesis could any 6\(\alpha\)-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 6\(\alpha\)-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 monophenols 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.

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-aminooazo-benzene could be demethylated by rat and mouse liver preparations in vitro to give p-aminooazo-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-aminooazo-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 $^{14}C$ 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 $^{14}C$ oestradiol-17β and rat liver homogenates. They obtained maximum binding in the presence of DPN$^+$, TPN$^+$ and an oxidizable substrate. ATP, flavin mononucleotide 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 $^{14}C$ 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 via the following sequence of reactions:

\[
\text{protein catechol} \rightarrow \text{o-quinone} \rightarrow \text{protein bound material}
\]

The reaction of quinones with -SH compounds is well known (Fieser and Fieser, 1950).

\[\text{O} + \text{HS.CH}_2\text{COOH} \rightarrow \text{25°C} \rightarrow \text{H}
\]

In this respect it is interesting that naphthalene can combine with glutathione via 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 $[^{14}C]$. 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 1 \( \mu \)g. of \( \left[ ^{14} \text{C} \right] \) oestradiol-17\( \beta \) of adding other steroids. A 55% inhibition was produced by 2 \( \mu \)g. of oestriol which he suggested was due to the oestriol competing with oestradiol-17\( \beta \) for the binding sites on the protein. No inhibition was produced by 0.1 \( \mu \)g. of 9a-fluoro-11\( \beta \)-hydroxyprogesterone, but at the 0.5 \( \mu \)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 \( \left[ ^{8} \text{C} \right] \) 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 intermediate muconic acid.

\[
\begin{align*}
&\text{COOH} \\
&\text{COOH} \\
&\text{COOH}
\end{align*}
\]

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 hydrocarbons 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 'a-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 $[^{14}C]$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 isocitric 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:

\[
\begin{align*}
\text{DPNH} & \rightleftharpoons \text{OESTRONE} & \text{TPNH} \\
\text{DPN}^+ & \rightleftharpoons \text{OESTRADIOL-17β} & \text{TPN}^+
\end{align*}
\]

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-17\(\beta\), 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, 17\(\alpha\)-ethinyloestradiol-17\(\beta\) and oestriol have no effect on this enzyme preparation. The latter compound may function via 16-oxo-oestradiol-17\(\beta\) (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-17\(\beta\) 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 17a-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 ↔ phenoxy radical equilibrium. They have produced evidence for the following sequence:

\[
\begin{align*}
2\text{ROH} + \text{O}_2 & \rightarrow 2\text{RO}^- + \text{H}_2\text{O}_2 \\
2\text{ROH} + \text{H}_2\text{O}_2 & \xrightarrow{\text{peroxidase}} 2\text{RO}^- + 2\text{H}_2\text{O} \\
4\text{RO}^- + 2\text{DPNH} + 2\text{H}^+ & \rightarrow 4\text{ROH} + 2\text{DPN}^+ \\
\text{net result} \quad 2\text{DPNH} + 2\text{H}^+ + \text{O}_2 & \rightarrow 2\text{DPN}^+ + 2\text{H}_2\text{O}
\end{align*}
\]
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 (McCorquodale 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 µm. All optical densities were read against a reagent blank.

<table>
<thead>
<tr>
<th>2-Hydroxyoestriol (µg.)</th>
<th>1</th>
<th>3</th>
<th>5</th>
<th>7.5</th>
<th>10</th>
<th>21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optical density (x 10^3)</td>
<td>25</td>
<td>77</td>
<td>132</td>
<td>232</td>
<td>328</td>
<td>698</td>
</tr>
</tbody>
</table>

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 µm. All optical densities were read against a reagent blank.
<table>
<thead>
<tr>
<th>2-Methoxyoestriol (µg.)</th>
<th>Optical Density (x 10^3)</th>
<th>2-Methoxyoestriol (µg.)</th>
<th>Optical Density (x 10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>88</td>
<td>20</td>
<td>336</td>
</tr>
<tr>
<td>7.2</td>
<td>123</td>
<td>25</td>
<td>427</td>
</tr>
<tr>
<td>10</td>
<td>167</td>
<td>30</td>
<td>524</td>
</tr>
<tr>
<td>15</td>
<td>252</td>
<td>50</td>
<td>912</td>
</tr>
<tr>
<td>18.2</td>
<td>305</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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).

<table>
<thead>
<tr>
<th>Time (hr.)</th>
<th>Oestriol Formed (%)</th>
<th>Time (hr.)</th>
<th>Oestriol Formed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubated under Air</td>
<td></td>
<td>Incubated under Nitrogen</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>10</td>
<td>0.5</td>
<td>6</td>
</tr>
<tr>
<td>1</td>
<td>14</td>
<td>1</td>
<td>18</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>3.5</td>
<td>16</td>
</tr>
</tbody>
</table>

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).

<table>
<thead>
<tr>
<th>Gas Phase</th>
<th>Oestriol Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen</td>
<td>55</td>
</tr>
<tr>
<td>Air</td>
<td>42</td>
</tr>
<tr>
<td>Oxygen</td>
<td>13</td>
</tr>
</tbody>
</table>

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).

<table>
<thead>
<tr>
<th>Additions</th>
<th>Oestriol Recovered</th>
<th>2-Methoxyoestriol Formed</th>
<th>2-Hydroxyoestriol Formed</th>
<th>Total Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>63</td>
<td>0</td>
<td>1</td>
<td>64</td>
</tr>
<tr>
<td>0.74 µM DPN$^+$ + 4.5 µM sodium lactate</td>
<td>51</td>
<td>4</td>
<td>1</td>
<td>56</td>
</tr>
<tr>
<td>1.48 µM DPN$^+$ + 9.0 µM sodium lactate</td>
<td>42</td>
<td>5</td>
<td>1</td>
<td>48</td>
</tr>
<tr>
<td>0.67 µM TPN$^+$ + 4.5 µM G-6-P</td>
<td>37</td>
<td>9</td>
<td>5</td>
<td>51</td>
</tr>
<tr>
<td>1.34 µM TPN$^+$ + 9.0 µM G-6-P</td>
<td>7</td>
<td>11</td>
<td>6</td>
<td>41</td>
</tr>
<tr>
<td>0.67 µM TPN$^+$ + 4.5 µM G-6-P + 0.74 µM DPN$^+$ + 4.5 µM sodium lactate</td>
<td>17</td>
<td>18</td>
<td>6</td>
<td>41</td>
</tr>
<tr>
<td>0.74 µM DPN$^+$ + 0.67 µM TPN$^+$</td>
<td>19</td>
<td>15</td>
<td>5</td>
<td>39</td>
</tr>
<tr>
<td>0.74 µM DPN$^+$ + 0.67 µM TPN$^+$</td>
<td>73</td>
<td>0</td>
<td>0</td>
<td>73</td>
</tr>
</tbody>
</table>

All incubations contained the following:
1 ml. of 20% homogenate in 0.25 M-sucrose plus
1.95 µM of oestriol in 0.035 ml. of PG plus 5 µM
of ATP plus 60 µM of L-methionine plus 150 µM
of MgCl₂ plus 240 µM of KCl plus 192 µM of KH₂PO₄,
pH 7.4. Final volume 3 ml. Incubated 1 hr.
under oxygen, 37°C.

<table>
<thead>
<tr>
<th></th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>150 µM MgCl₂</td>
<td>20</td>
</tr>
<tr>
<td>150 µM MgCl₂ +</td>
<td>25</td>
</tr>
<tr>
<td>60 µM L-</td>
<td>23</td>
</tr>
<tr>
<td>methionine</td>
<td></td>
</tr>
<tr>
<td>150 µM MgCl₂ +</td>
<td>33</td>
</tr>
<tr>
<td>5 µM ATP</td>
<td>38</td>
</tr>
<tr>
<td>150 µM MgCl₂ +</td>
<td>30</td>
</tr>
<tr>
<td>5 µM ATP + 50</td>
<td>30</td>
</tr>
<tr>
<td>µM L-methionine</td>
<td></td>
</tr>
<tr>
<td>150 µM MgCl₂ +</td>
<td>49</td>
</tr>
<tr>
<td>5 µM ATP + 50</td>
<td>48</td>
</tr>
<tr>
<td>µM L-methionine</td>
<td></td>
</tr>
<tr>
<td>Oestriol added</td>
<td>78</td>
</tr>
<tr>
<td>just prior to</td>
<td></td>
</tr>
<tr>
<td>extraction</td>
<td>78</td>
</tr>
</tbody>
</table>

All incubations used the following:
1 ml. of 20% homogenate in 0.25 M-sucrose plus
0.74 µM PG + 1 µM of TMR* plus 1.75 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.
2. Effect of Mg$^{2+}$, Ca$^{2+}$, ATP and L-methionine on 2-methoxyoestriol and 2-hydroxyoestriol formation by rat liver homogenates (Fig. 8b).

<table>
<thead>
<tr>
<th>Oestriol Recovered</th>
<th>2-Methoxyoestriol Formed</th>
<th>2-Hydroxyoestriol Formed</th>
<th>Total Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>150 µM MgCl$_2$</td>
<td>20</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>150 µM MgCl$_2$ + 60 µM L-methionine</td>
<td>25</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>150 µM MgCl$_2$ + 5 µM ATP</td>
<td>23</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>150 µM CaCl$_2$ + 5 µM ATP + 60 µM L-methionine</td>
<td>30</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>150 µM MgCl$_2$ + 5 µM ATP + 60 µM L-methionine. Oestriol added just prior to extraction.</td>
<td>19</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>150 µM MgCl$_2$ + 5 µM ATP + 60 µM L-methionine. Oestriol added just prior to extraction.</td>
<td>73</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

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 KH$_2$PO$_4$, 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).

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

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).

<table>
<thead>
<tr>
<th>Additions</th>
<th>Oestriol Recovered</th>
<th>2-Hydroxy-oestriol Formed</th>
<th>Total Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ml. microsome + supernatant fraction. Oestriol added just prior to extraction.</td>
<td>74</td>
<td>-</td>
<td>74</td>
</tr>
<tr>
<td>1 ml. microsome + supernatant fraction + 4.5 µM Na lactate + 4.5 µM G-6-P</td>
<td>43</td>
<td>13</td>
<td>56</td>
</tr>
<tr>
<td>1 ml. preincubated microsome + supernatant fraction + 4.5 µM Na lactate + 4.5 µM G-6-P</td>
<td>49</td>
<td>13</td>
<td>62</td>
</tr>
<tr>
<td>1 ml. preincubated microsome + supernatant fraction</td>
<td>53</td>
<td>8</td>
<td>61</td>
</tr>
</tbody>
</table>

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).

<table>
<thead>
<tr>
<th>Additions</th>
<th>Oestriol Recovered %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate minus debris and nuclei</td>
<td>3</td>
</tr>
<tr>
<td>((\equiv) 100 mg. wet weight whole liver)</td>
<td></td>
</tr>
<tr>
<td>Mitochondria ((\equiv) 340 mg. wet weight whole liver)</td>
<td>69</td>
</tr>
<tr>
<td>Microsomes ((\equiv) 340 mg. wet weight whole liver)</td>
<td>29</td>
</tr>
<tr>
<td>Supernatant ((\equiv) 100 mg. wet weight whole liver)</td>
<td>60</td>
</tr>
<tr>
<td>Boiled microsomes</td>
<td>78</td>
</tr>
<tr>
<td>Microsomes. Oestriol added just prior to extraction</td>
<td>79</td>
</tr>
</tbody>
</table>

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 \(\mu\)g. of oestriol in 0.05 ml. of PG. Incubated 2 hr. in air at 37\(^\circ\)C.
APPENDIX III

PREPARATION OF MATERIALS

1. SOLVENTS


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

The author wishes to express his thanks to Dr G.F. Marrian, F.R.S., for his kind advice and guidance in these researches; to Dr J.K. Grant who gave much helpful advice; to Dr J.W. Minnis who weighed out materials and carried out micro-analyses; to Dr M.M. Coombs of the Imperial Cancer Research Fund Laboratories, who very kindly undertook the synthesis of 2-hydroxy-oestriol and also determined some of the infrared spectra; to Dr R.K. Callow, F.R.S., of the National Institute for Medical Research and Dr T.F. Gallagher of the Sloan-Kettering Institute for Cancer Research, for their infrared determinations; and to Dr E.J.D. Sinclair for reading through the manuscript prior to publication.
REFERENCES


