Radioactive estradiol-17α was identified in the urines of hens that had received injections of tracer amounts of estradiol-17β-4-14C and/or estrone-4-14C. Estradiol-17α was a major urinary conversion product of injected estrone-4-14C in the non-laying as well as the laying hen. 16-Ketoestrone was found not to be a major urinary conversion product, if indeed it was present in the urine. Injected estradiol-17α gave rise to radioactive urinary estrone and probably also to other urinary steroid estrogens. When radioactive estrone was injected into a hen during the non-laying and the laying state, the relative proportions of urinary radioactive estradiol-17β, estradiol-17α and estrone differed as between the non-laying and the laying state. The ratios of these estrogens were 7.1 : 1 : 1.5 and 1 : 1 : 1.5 for the non-laying and the laying hen respectively.
SHORT TITLE:

OCCURRENCE OF ESTRADIOL-17α IN HEN'S URINE.

SHREE MULAY
SHORT TITLE:

OCCURRENCE OF ESTRADIOL-17\alpha IN HEN'S URINE.

SHREE MULAY
ON THE OCCURRENCE
AND SIGNIFICANCE OF ESTRADIOL-17α AS A URINARY CONVERSION
PRODUCT OF INJECTED ESTRONE OR ESTRADIOL-17β IN THE
DOMESTIC FOWL

A Thesis
by
Shree Mulay

Submitted to the Faculty of Graduate Studies and Research,
McGill University, in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

Department of Agricultural Chemistry,
McGill University,
ACKNOWLEDGEMENTS

The author wishes to thank Professor R.H. Common who suggested the research described in this thesis and provided much encouragement throughout its course.

The author is indebted to the following for their help.

Professor G.O. Henneberry, Macdonald College for helpful advice concerning liquid scintillation counting.

Dr. R.S. Mathur, Macdonald College, for operating the experimental birds and for his invaluable advice on several occasions during the course of this research.

Dr. N. Nikolaiczuk, Macdonald College, for the provision of experimental birds.

Dr. G. Tang, Mr. P. Chen and Mr. R. Robinson, Macdonald College, for their assistance with the experimental birds.

Mr. P. Frey, Macdonald College, for printing some of the photographs for this thesis.

Mr. H.F. Johnston, formerly at Macdonald College, for his help with the preliminary experiment done in connection with the identification of estradiol-17α.

Mr. G.L. Lancaster, formerly at Macdonald College, for performing the bacterial counts on the urine samples.

The author also wishes to thank Dr. J. Fishman, Montefiore Hospital, New York, N.Y. and Dr. D.S. Layne for their gift of reference steroids.

In conclusion, the author wishes to thank the National Research
Council of Canada for financial support provided by the award of a Graduate Scholarship held by the author throughout the course of this research; and thank the National Institute of Arthritis and Metabolic Diseases the National Institutes of Health, U.S.P.H. and the National Research Council of Canada for providing the general financial support of this work.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>LIST OF SYSTEMATIC NAMES OF STEROID ESTROGENS</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>GENERAL INTRODUCTION</td>
<td>1</td>
</tr>
</tbody>
</table>

## CHAPTER I

### HISTORICAL REVIEW

**Introduction** ............................................................. 3

**A. Occurrence and metabolism of estrogens in humans**

1. Introduction ............................................................. 4
2. Isolation of estrogens ................................................. 5
3. Identification of estrogens as *in vivo* metabolites .......... 11
4. Identification of estrogens as *in vitro* metabolites ......... 17
5. Metabolism of estrogens in humans .................................. 21

**B. Occurrence of estrogens in certain mammalian species other than humans**

1. Introduction ............................................................. 33
2. Isolation of estrogens ................................................. 33
3. Identification of estrogens as *in vivo* metabolites .......... 39
4. Identification of estrogens as *in vitro* metabolites ......... 44

**C. Occurrence and metabolism of estrogens in certain avian species**

1. Introduction ............................................................. 51
2. Isolation of estrogens ................................................. 52
3. Identification of estrogens as *in vivo* metabolites .......... 56
4. Identification of estrogens as *in vitro* metabolites ......... 60
5. Metabolism of estrogens in avian species ......................... 65

**D. Occurrence of estrogens in vertebrates other than mammals and aves** ........................................... 70

## CHAPTER II

### MATERIALS AND METHODS

**A. General materials and apparatus**

1. Reference standards .................................................... 71
2. Solvents ........................................................................... 72
3. Chemicals ......................................................................... 73
4. Radiochemicals and materials for counting 73
5. Enzyme preparations 74
6. Colour reagents 74
7. Chromatographic equipment 75

B. Experimental procedures
1. Collection and storage of urine samples
   a. In vivo experiments 75
   b. Ad hoc experiments 76
2. Filtration 76
3. Enzymatic
   a. Hydrolysis with ketodase 76
   b. Hydrolysis with sulfatase 76
4. Solvolysis 77
5. Thin layer chromatography
   a. Preparation of thin-layer plates 77
   b. Solvent systems 78
   c. Visualization of steroid estrogens 78
   d. Application of sample on thin-layer plates 81
6. Measurement of radioactivity
   a. Measurement of radioactivity in urine 81
   b. Measurement of radioactivity in methanolic extract 82
   c. Assay of radioactivity on thin-layer plates 82
7. Radioautography 83
8. Separation of ketonic and non-ketonic estrogens 84
9. Preparation of derivatives
   a. Acetylation 84
   b. Methylation 85
10. Identification of presumptive estrogen by reverse isotope dilution 85

CHAPTER III

PRELIMINARY AD HOC EXPERIMENTS

A. Experiment I
1. Object 87
2. Experimental 87
3. Results and discussion 87
B. Experiment II
1. Object ................................................ 89
2. Experimental ........................................... 89
3. Results and discussion ................................. 91

C. Experiment III
1. Object ................................................ 94
2. Experimental ........................................... 94
3. Results and discussion ................................. 95

CHAPTER IV
IDENTIFICATION OF ESTRADIOL-17α AS AN IN VIVO CONVERSION PRODUCT OF ESTRADIOL-17β-4-14C AND ESTRONE-4-14C
A. Introduction ............................................. 98
B. Experimental and results
1. Experiment I ............................................ 100
   a. Outline of procedure for processing urine .......... 100
   b. Results ........................................ 101
2. Experiment II ......................................... 103
3. Experiment III ........................................ 108
   a. Procedure for processing urine ...................... 109
   b. Results ........................................ 110
4. Ad Hoc Experiment ..................................... 115
C. Discussion ............................................. 117

CHAPTER V
IN VIVO CONVERSION OF ESTRADIOL-17α-6,7-3H TO URINARY ESTRONE BY THE HEN
A. Introduction ............................................. 121
B. Experimental and results
1. Experimental ........................................... 121
2. Results ............................................... 123
   a. Zone corresponding to reference estrone .......... 126
   b. Zone corresponding to reference estradiol-17α, estradiol-17β, 16-ketoestrone and 16-ketoestradiol-17β ....... 129
   c. Zone corresponding to reference 16-epiestriol and 17-epiestriol ........................................ 132
Chapter VI

A study of some conversion products of estrone-4-$^{14}$C in the laying and the non-laying hen with special reference to the status of 16-ketoestrone as a urinary steroid estrogen

A. Introduction .................................................. 138
B. Experimental ............................................... 140
C. Results and discussion ................................. 142

Summary ............................................................. 153

Claims to original research .................................. 156

Publications by the candidate ............................ 158

Bibliography ..................................................... 159

List of tables

I. Estrogens isolated and identified from human pregnancy urine .......................... 7
II. Estrogens identified in non-pregnancy urine, male urine and various organs ........ 9
III. Steroid estrogens isolated and identified in the hormone producing tissue of certain mammalian species .................................................. 35
IV. Steroid estrogens isolated and identified from the urine of certain mammalian species other than humans ........................................ 37
V. Isolation of estrogens in crystalline form from avian excreta ......................... 54
VI. Other isolations of estrogens from avian material ..................................... 55
VII. Conversion of phenolic steroids by chicken liver in vitro ............................. 62
VIII. Composition of solvent systems used for thin-layer chromatography ............... 79
IX. Proportion of radioactivity in zones corresponding to reference estrogens

X. Experiment III: Proportions of radioactivity in zones corresponding to reference estrogens

XI. Experiment I: Chromatographic mobilities of urinary presumptive estradiol-17α and estradiol-17β and their derivatives and mobilities of corresponding reference material

XII. Experiment II: Recoveries of radioactivity in the urine and certain urinary fractions following the injection of estrone-4-14C into a non-laying hen

XIII. Experiment II: Crystallization of urinary presumptive estradiol-17α to constant specific activity with carrier

XIV. Experiment II: Chromatographic mobilities of urinary presumptive estradiol-17α and its derivatives

XV. Experiment III: Recoveries of radioactivity in the urine and certain urinary fractions following the injection of a mixture of estrone-4-14C and estradiol-17β-4-14C into a laying hen

XVI. Experiment III: Crystallizations of urinary presumptive estradiol-17α and its diacetate to constant specific activity with carrier

XVII. Experiment III: Chromatographic mobilities of urinary presumptive estradiol-17α and its derivatives

XVIII. Recoveries of injected radioactivity in the urine

XIX. Recoveries of radioactivity in urinary fractions after hydrolysis

XX. Crystallization of radioactive urinary presumptive estrone to constant specific activity with reference estrone

XXI. Chromatographic mobilities of radioactive urinary estrone –3H and its acetate

XXII. Recoveries of radioactivity in the urines and certain urinary fractions from a hen that received estrone-4-14C in the non-laying phase and the laying phase

XXIII. Percent distribution of urinary conversion products of injected estrone for the laying and non-laying hen
XXIV Proportions of non-ketonic and ketonic material in the presumptive estradiol-17α and/or 16-ketoestrone obtained by chromatography of urinary fractions 147

LIST OF FIGURES

I Metabolic scheme of estrogens by Pincus and Pearlman (1943) 21
II Metabolic scheme of estrogens by Heard (1949) 22
III Intermediary metabolism of C-16 substituted estrogens in humans. Intermediary metabolism of C-2, C-6, C-15 and C-18 substituted estrogens in humans 31
IV Intermediary metabolism of estrogens in the domestic fowl 68
V Photograph illustrating the relative mobilities of reference methyl ethers and acetates of the two estradiol epimers and the acetates of estrone, estriol and 16-ketoestrone in System Z. 80
VI Experiment III: Radioautogram of chromatoplate of non-ketonic fraction of phenolic extract 113
VII Experiment III: Photographs illustrating the complete correspondence of presumptive radioactive material with reference estradiol-17α 115a
VIII Thin-layer chromatogram (System X) of estradiol-17α-6,7-3H used for injections. 122
IX Distribution of radioactivity on thin-layer chromatogram (System X) of CHCl₃ extracts of enzyme hydrolyzed urine 128
X Photograph illustrating the complete correspondence between the radioactive peak and colouration due to reference estrone 131
XI Distribution of radioactivity on thin-layer chromatogram (System X, double development) of non-ketonic fraction from the major peak obtained by TLC of urinary CHCl₃ extracts 133
XII Distribution of radioactivity on thin-layer chromatogram (System X) of CHCl₃ extracts of urine control from uninjected bird 135
XIII Percentage distribution of urinary phenolic conversion product of injected estrogen 151
Nomenclature of estrogens

<table>
<thead>
<tr>
<th>Trivial</th>
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<tr>
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<td>3-hydroxy-estra-1,3,5(10)-triien-16,17-dione.</td>
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<td>16-Ketoestradiol-17β</td>
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<td>Estriol</td>
<td>Estra-1,3,5(10)-triene-3,16α,17β-triol.</td>
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<tr>
<td>16-Epiestriol</td>
<td>Estra-1,3,5(10)-triene-3,16β,17β-triol.</td>
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<td>16,17-Epiestriol</td>
<td>Estra-1,3,5(10)-triene-3,16β,17α-triol.</td>
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<td>Estra-1,3,5(10)-triene-3,16α,17α-triol.</td>
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<td>Equilin</td>
<td>3-hydroxy-estra-1,3,5(10)-7(8)-tetraene-17-one.</td>
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<tr>
<td>Equilenin</td>
<td>3-hydroxy-estra-1,3,5(10)-6,8(9)-pentaene-17-one.</td>
</tr>
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GENERAL INTRODUCTION

It has been well established that the profound changes in the physiology and morphology of a normal pullet at puberty are induced by hormones elaborated by the hen and that steroid estrogens play a central role in the physiology and biochemistry of avian reproduction.

Although a great deal was known about the physiological effects of estrogens in hens, the nature of estrogens produced by them remained obscure until 1957, when two reports were published on the nature of endogenous estrogens in hens. Hurst (1957) at Queens University, Kingston, reported the identification of estrone and estriol in the droppings of hens and the identification of estrone, estradiol-17β and estriol in the droppings of roosters. Layne, at Macdonald College, on the other hand, reported the detection of estrone, estradiol-17β and, with less certainty, estriol in the ovaries of laying hens (Layne, 1958).

Following the publication of these two reports, the group at Macdonald College undertook a systematic investigation of the estrogens elaborated by the hen and the in vivo interconversions of these estrogens. These studies, extending over a period of several years, have resulted in the isolation and identification of several estrogens from the urine of the hen; the estimation of the amounts of urinary estrone, estradiol-17β and cis-estriols excreted in 24-hours in the laying and non-laying hens; and have established the interconversions of estrogens effected by the hen.

Breuer in West Germany and Hobkirk in Montreal have further contributed to the knowledge of estrogen metabolism in the domestic fowl based on in vitro studies. Recently, Renwick and Engel (1967) have been successful in partially purifying the enzymes implicated in the estrogen conversions in
the hen. O'Grady and Heald (1965) have also reported the identification of estrone and estradiol-17β in the avian blood plasma.

These findings have been described in the appropriate section of this thesis. It is evident that considerable knowledge regarding the estrogens elaborated by the hen and the metabolism of those estrogens has accumulated in the last decade.

The present thesis is concerned with the extension of knowledge regarding the nature of in vivo conversion products of estrone, estradiol-17β and estradiol-17α and the differences in the distribution pattern of estrogen metabolites in the laying and non-laying hens. This thesis, therefore, includes:

a) The identification of estradiol-17α as an in vivo urinary conversion product of estrone and estradiol-17β.

b) The identification of estrone as one of the in vivo urinary conversion product of estradiol-17α.

c) A comparison of the distribution pattern of the in vivo conversion products of labelled estrone in the laying and non-laying hens.

d) The demonstration that estradiol-17α is a major urinary conversion product in the laying and non-laying hens.
CHAPTER I

HISTORICAL REVIEW
INTRODUCTION

A great deal of knowledge concerning estrogens, their physiological activity, chemical structure, biogenesis, intermediary metabolism, inactivation and conjugation has been acquired over the past seventy years. The emphasis of most of these studies has been in relation to the human subject, first, because the discovery that a readily available source of steroid estrogens, pregnancy urine, was extremely rich in estrogens led to the isolation of a large number of estrogens. Second, the elucidation of the relationship between the excretion of estrogens and ovulation stimulated efforts to control fertility by interfering with the pituitary-ovary relationship. Third, the observation that the excretion of high levels of estrogens is correlated to the viability of the fetus in a pregnancy has led to the study of the feto-placental unit.

At the same time, an appreciable amount of research has been done in the study of other animal species, more particularly over the last twenty years, from the viewpoint of comparative endocrinology and in an attempt to understand more completely the endocrinology of various mammals so that their usefulness as experimental animals for studying drugs, etc., is enhanced.

Since the present thesis is concerned with metabolism of estrogens in vivo in the fowl, the historical review will be directed mainly to the material concerning the occurrence and metabolism of estrogens. No attempt will be made to cover even these topics comprehensively, since the literature concerning this subject is vast and a number of excellent reviews are available.
A. OCCURRENCE AND METABOLISM OF ESTROGENS IN HUMANS

1. INTRODUCTION

The earliest studies in which the estrus cycle was directly related to an active principle from the female gonads were done in the first decade of the twentieth century. This active principle was shown to be of a lipid nature. In 1923 Allen and Doisy came to the tentative conclusion that the physiological activity was due to a single chemical compound. A search for this compound was undertaken by several groups which resulted in the isolation of estrone from pregnancy urine, between 1929-1930, by three independent groups. A few years later a second estrogen, estriol was obtained from the same source. By 1939 yet another estrogen, estradiol-17β, was discovered. The discovery that these hormones were steroidal in nature stimulated the study of their interrelationships with other steroids. The fact that more than one estrogenic hormone existed naturally suggested that the most potent hormone may have been metabolized to less potent estrogens and vice-versa. Elucidation of the metabolism of estrogen was done by administering large doses of pure estrogens and examining the urine for the metabolites. This type of experiment became possible only when large amounts of estrogens became readily available in the late thirties.

Phenolic steroids which are physiologically very active occur in very small amounts as compared to the neutral steroids and the corticosteroids. Thus the isolation of metabolites of estrogens other than the classical estrogens was difficult in view of the methodological problems associated with purification and identification. There was a quiescent period during
which no new estrogens were discovered. Over the 20 years following the discovery of the "classical estrogens", chromatography developed into a sophisticated technique which was both sensitive and specific in the separation of steroid estrogens. Physical methods for identification became available, which required micro amounts of the compound for identification. These methods included ultraviolet spectroscopy, mass spectrometry, infrared spectroscopy, X-ray crystallography, nuclear magnetic resonance and gas chromatography. These methods provided the proof for the structure of steroid under study. The Kober reaction was refined by Brown (1952), Bauld (1954), and Ittrich (1960) and the accuracy of the spectrophotometric measurements involved was improved by application of the Allen correction (Allen, 1950), while fluorimetric methods were also introduced. These advances hastened the identification of hitherto unrecognized phenolic steroids.

A large number of the "newer estrogens" were discovered by Marrian's group in Edinburgh and by Breuer's group in Bonn. The literature up until 1961 has been reviewed by Breuer (1961), Diczfalusy and Lauritzen (1961) and Adlercreutz (1962). Brown (1965) and Morand and Lyall (1968) have covered the more recent period.

2. ISOLATIONS

The organs involved in the production of estrogens in humans have been shown to include the ovaries, the testes, the adrenals, corpora lutea, placenta and the fetal liver. The fetal adrenal has been shown to produce the precursor for estrogens, which is converted to estrogens by the fetal liver and the placenta. Estrogens have been isolated from the urine and most often from pregnancy urine which contains large amounts of estrogens.
The estrogens isolated from pregnancy urine are tabulated in Table I. The estrogens are listed in the chronological order of discovery. Table II enumerates the estrogens isolated and identified from non-pregnancy urine, male urine and various organs.

There are two reports on the identification of estradiol-17α in the human. Adlercreutz and Luukkainen (1965) detected eight different metabolites of estrogens in the late pregnancy bile, using gas chromatography. The following estrogens were identified in the glucosiduronate fraction: - estriol, 16-epiestriol, 16,17-epiestriol, 17-epiestriol, estradiol-17β, 16-ketoestradiol-17β, 16 α-hydroxyestrone and estradiol-17α. The authors, however, point out that no definite conclusions, except for estriol, can be drawn about the presence of these estrogens in the late pregnancy bile. The second report concerning estradiol-17α is by Schott and Katzman (1964). They identified estradiol-17α in the urine of one pregnant subject only. In all other subjects studied, estradiol-17α was not identified. The subject in whose urine estradiol-17α was identified had a history of endocrine disturbances, therefore estradiol-17α cannot be considered as a normal metabolite of estrogen in the human subject. Earlier attempts by Marrian (1958) to identify estradiol-17α in pregnancy urine were fruitless.

Luukkainen and Adlercreuetz (1965) have also reported the isolation and identification of 11-dehydro-17α-estradiol from human pregnancy urine.

Another estrogen which was isolated from pregnancy urine but not identified rigorously is the compound referred to as KC-6B by Loke, Marrian and Watson (1959). This compound is believed to be 6-hydroxyestrone (Marrian, 1961).
TABLE I

Estrogens isolated and identified from human pregnancy urine

<table>
<thead>
<tr>
<th>Estrogen</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Estrone</td>
<td>Doisy, Veler &amp; Thayer (1929); Butenandt (1929)</td>
</tr>
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<td></td>
<td>Dingemanse, deJongh, Kober &amp; Laqueur (1930)</td>
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<td>Estriol</td>
<td>Marrian, (1930); Doisy &amp; Thayer (1931)</td>
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<tr>
<td>Estradiol-17β</td>
<td>Huffman, MacCorquodale, Thayer, Doisy, Smith &amp; Smith (1940)</td>
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<td>16-Epiestriol</td>
<td>Marrian &amp; Bauld (1954, 1955)</td>
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<td>16α-Hydroxyestrone</td>
<td>Marrian, Loke, Watson &amp; Panattoni (1957)</td>
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<td>Layne &amp; Marrian (1958a,b)</td>
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<td>2-Methoxyestrone</td>
<td>Loke &amp; Marrian (1958)</td>
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<td>Layne &amp; Marrian (1958b)</td>
</tr>
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<td>18-Hydroxyestrone</td>
<td>Loke, Marrian, Johnson, Meyer &amp; Cameron (1958); Loke, Marrian &amp; Watson (1959)</td>
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<td>2-Methoxyestradiol-17β*</td>
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<td>16,17-Epiestriol</td>
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</tr>
<tr>
<td>17-Epiestriol</td>
<td>Breuer &amp; Pangels (1961)</td>
</tr>
<tr>
<td>Estrogen</td>
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<td>2-Hydroxyestrone</td>
<td>Notchev &amp; Stimmel (1962)</td>
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<td>15α-Hydroxyestrone</td>
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</table>

* * denotes tentative identification

Note: Prior to 1950, before the absolute configuration of the hydroxyl groups on estrogen molecules were determined, estra-1,3,5 (10)-trien-3,17β-diol was known by the trivial name α-estradiol. Similarly, its epimer estra-1,3,5 (10)-trien-3,17α-diol was known as β-estradiol.
TABLE II

Estrogens isolated and identified in non-pregnancy urine, male urine, and various organs.

<table>
<thead>
<tr>
<th>Estrogen</th>
<th>Source</th>
<th>References</th>
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<td>Westerfeld, MacCorquodale, Thayer &amp; Doisy (1938).</td>
</tr>
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<td></td>
<td>Male urine</td>
<td>Dingemanse, Laqueur, Muhlbock (1938).</td>
</tr>
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<td></td>
<td>Feminizing adrenal tumor</td>
<td>Mahesh &amp; Herrmann (1963)</td>
</tr>
<tr>
<td>Estradiol-17β</td>
<td>Feminizing testicular tumor</td>
<td>Marti &amp; Heusser (1954)</td>
</tr>
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<td>Normal testes</td>
<td>Goldzieher &amp; Roberts (1952)</td>
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<td>Ovaries</td>
<td>Zander et al. (1959), Smith (1960)</td>
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<td>Engel et al. (1952)</td>
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<tr>
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<td>Source</td>
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<td>Feminizing adrenal tumor</td>
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<td>Diczfalusy &amp; Halla (1958)</td>
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</tr>
<tr>
<td>16α-Hydroxyestrone</td>
<td>Non-pregnancy urine</td>
<td>Loraine (1958)</td>
</tr>
<tr>
<td>Estranediol A</td>
<td>Non-pregnancy urine</td>
<td>Marker, Rohrmann, Lawson &amp; Wittle (1938)</td>
</tr>
<tr>
<td>Estranediol B</td>
<td>Non-pregnancy urine</td>
<td></td>
</tr>
</tbody>
</table>

* denotes tentative identification
3. IDENTIFICATION OF ESTROGENS AS IN VIVO METABOLITES

Up until 1955 only three naturally occurring estrogens had been discovered in the human. A considerable amount of interest centered around the interconversion of these estrogens. Smith and Smith (1938) found both estradiol-17β and estriol in the urine of women injected with estrone. Pincus and Pearlman (1942) demonstrated that estrone was converted to estradiol-17β and estriol in about 0.1% yield in both men and women. Heard and Hoffman (1941) were able to demonstrate the opposite reaction, i.e., conversion of estradiol-17β to estrone in man. Schiller and Pincus (1943), by application of partition methods, were able to demonstrate that 2.7% of estradiol-17β was converted to estrone and estriol. They were however, unsuccessful in demonstrating the conversion of estriol to estrone or estradiol-17β in man. It was believed, therefore, that reactions leading to the formation of estriol were irreversible in the human species.

With the advent of radioactive tracer technique and the synthesis of isotopically labelled estrogens where the label had been introduced in ring A or ring D it was possible to detect minute amounts of an estrogen metabolite. Tracer doses of labelled estrogen were administered followed by characterization of the labelled product. Reverse isotope dilution and crystallization to constant specific activity proved of great value in establishing the identity of the steroid. ¹⁴C-Labelled estrogens and, more recently, ³H-labelled estrogens are preferred since these can be used for double label counting.
Many C-16 substituted metabolites have been isolated and identified. Some of these were first identified as in vivo metabolic products of exogenous estrogens. Estriol was the first C-16 substituted estrogen to be discovered. Other reports concerning the conversion of estrone and estradiol-17β to estriol will be described in the metabolism section along with the kinetic studies, and will not be described here. The formation of 16-epiestriol from estradiol-17β has been amply demonstrated both in vivo and in vitro. Brown, Fishman and Gallagher (1958) and Engel, Cameron, Stoffyn, Alexander, Klein and Trofimow (1961) detected labelled 16-epiestriol in the urine upon administration of estradiol-17β. Similarly, 16-epiestriol was detected as a conversion product of estradiol-17β-6,7-3H in the urine after injection into men (Hobkirk, Nilsen and Purre, 1966) and into women in the last stages of pregnancy (Hobkirk and Nilsen, 1966).

The formation of triols from estrone requires 16α-, or 16β-hydroxylation. Accordingly, Brown, Fishman and Gallagher (1958) reported the isolation of 16β-hydroxyestrone-16-14C from urine following the injection of estradiol-17β-16-14C into a nonpregnant subject, at the same time as its isolation from pregnancy urine by Layne and Marrian (1958a,b). More recently, Hobkirk et al. (1966) and Hobkirk and Nilsen (1966) have detected both 16α-hydroxyestrone-3H and 16β-hydroxyestrone-3H from the urine of men and women following injection of estradiol-17β-6,7-3H.

Further conversion of 16α-hydroxyestrone was studied by Brown and Marrian (1957) who injected 16α-hydroxyestrone into two individuals and were able to identify estriol in the urine. Nocke, Breuer and Knuppen
(1961) isolated estriol and 17-epiestriol from the urine following injection of 16α-hydroxyestrone into a male subject. Furthermore, they identified 16-epiestriol and 16,17-epiestriol in the urine after injection of 16β-hydroxyestrone.

Levitz, Spitzer and Twombly (1956) detected radioactive 16-ketoestradiol-17β in non-pregnancy urine following the administration of estradiol-17β-16-14C. More recently Hobkirk et al. (1966) and Hobkirk and Milsen (1966) have identified radioactive 16-ketoestradiol-17β as a conversion product of estradiol-17β-6,7-3H.

Levitz et al. (1958) injected estriol-16-14C into two women and detected very small but significant amounts of labelled 16-ketoestradiol-17β and 16-epiestriol in the urine. The rest of the activity occurred as unchanged estriol. This conversion indicated that enzymes capable of oxidizing the 16-hydroxyl group are present in humans. These experiments could not at first be duplicated by other investigators. More recently, Fishman, Zumoff, Hellman and Gallagher (1968) studied the metabolism of estriol, stereospecifically labelled with tritium in the 17α-position. A mixture of estriol-17α-3H and estriol-4-14C was administered to three subjects. No tritium label was identified in the plasma or urine water. The greater part of the radioactivity excreted consisted of estriol which had not lost any tritium label at the 17α-position. The only other compounds identified were 16-ketoestradiol-17β (0.8%) and 16-epiestriol(0.2%). Both of these compounds retained the tritium label in the 17α-position, hence these conversion products of estriol could not have arisen via a 17-keto intermediate. The in vivo conversion of 16-epiestriol-16-14C was demonstrated by Hobkirk

The presence of 16-ketoestrone in the urine from pregnant women who had received estrone-16-14C has been reported by Slaunwhite and Sandberg (1956). Migeon, Wall and Bertrand (1959) have also detected 16-ketoestrone as a conversion product of estrone-16-14C based on chromatographic evidence. Other estrogens detected by them are estriol, 16-epiestriol, 16α-hydroxyestrone and 16-ketoestradiol-17β. Serchi (1953), on the other hand, reported the identification of 16-ketoestrone from non-pregnancy urine. However, in none of these reports has the identification of 16-ketoestrone been rigorous. This task of identification of 16-ketoestrone is made further difficult by the extreme lability of the compound. Beling, Breuer and Breuer (1963) reported the isolation and identification of 16-ketoestrone in a crystalline form from urine of normal subjects following the injection of 16-ketoestradiol-17β. Following the injection of 16-ketoestrone into man, Stimmel, Grollman and Huffman (1948, 1950) showed the presence of estriol and 16-epiestriol (Stimmel, 1958) in the urine. 16-Ketoestradiol-17β was apparently formed as an intermediate (Stimmel, 1958).

In addition to C-16 substituted phenolic steroids a number of C-2 substituted phenolic steroids have been identified as in vivo metabolites of exogenous estrogens and these are by no means quantitatively minor metabolites of estrogens. 2-Methoxyestrone (Kraychy and Gallagher, 1957a, 1957b; Engel, Baggett and Carter, 1957), 2-methoxyestriol (Fishman and
Gallagher, 1958) and 2-hydroxyestrone (Fishman, Cox and Gallagher, 1960) have been identified as in vivo conversion products of estradiol-17β-16-14C in the urine of normal subjects. Axelrod, Rao and Goldzieher (1960, 1961) identified 2-methoxyestriol, 2-methoxyestrone and 2-methoxyestradiol-17β in normal urine following the infusion of 2-hydroxyestradiol-17β into post-menopausal women. Demethylation of the C-2 and C-3 substituted estrogens has been demonstrated in vivo by Hobkirk and Nilsen (1963) and Breuer, Knuppen, Gross and Mittermayer (1964) respectively.

Several other estrogen metabolites which are apparently of minor significance both quantitatively and physiologically have been detected in the urine. Of these, C-11 substituted estrogens have not been detected as a radioactive metabolites of exogenous estrogens. The only report concerning C-11 substituted estrogen is by Chang and Dao (1961, 1962). They injected cortisone-4-14C acetate into castrated, adrenalectomized women with metastatic breast cancer and were able to identify 11β-hydroxyestrone and 11β-hydroxyestradiol-17β in the urine.

Of late there has been a considerable amount of interest in the C-6 substituted phenolic steroids following the discovery that mouse and rat liver microsomes can convert estradiol-17β-16-14C to the 6-hydroxylated compounds. Breuer and Breuer (1965) injected 10 mg. of 6-ketoestrone intramuscularly into normal individuals and identified the following metabolites in the order decreasing amounts in the urine: 6α-hydroxyestrone, 6-hydroxyestrone, 6α-hydroxyestradiol-17β, 6β-hydroxyestradiol-17β, 6-ketoestrone and 6-ketoestradiol-17β. Up until recently it was believed that C-6 substituted metabolites did not arise endogenously, at least,
Breuer et al. (1966) and Knuppen et al. (1966b) isolated and identified 6-hydroxyestriol and 6α-hydroxyestrone, respectively, from pregnancy urine. Breuer et al. (1966) were unable to establish orientation of the 6-hydroxy group of the 6-hydroxyestriol.

Many reports on the tetrols and other unusual phenolic steroids have appeared in the literature in recent years. Hagen, Barr and Diczfalusy (1965) injected estradiol-17β-16-14C into three infants with multiple malformations and they found a compound more polar than 2-hydroxyestriol in the urine. This fraction accounted for 16% of the total urinary estrogens present. They have suggested that this compound is a tetrol with either two hydroxyl groups in the 16α- and 15α- positions or in 16α- and 18- positions. Following the intra-amniotic injection of tritiated estriol and estradiol-17β-4-14C into a woman at the 14th week of gestation, Schwers, Gurpide, Wiele and Lieberman (1967) identified the following metabolites: estrone, estradiol-17β, 16-epiestriol, estriol, 16-ketoestradiol-17β and a tetrol from 64hr urine. The isotopic ratios of these metabolites showed that both the injected compounds were converted to the tetrol and also suggested that conversion of estradiol-17β to the tetrol occurred only partially via estriol. The most abundant urinary metabolite derived from estradiol-17β was the tetrol. This tetrol has since been isolated from pregnancy urine and identified as 15α-hydroxyestriol (Zucconi et al., 1967).

Since the discovery that bovine adrenals were capable of hydroxylating estrogens in the 15α- position (Knuppen and Breuer, 1964), a number of C-15 substituted metabolites have been isolated from human pregnancy urine and identified as listed in Table I. Schwers, Eriksson and Diczfalusy (1965)
and Schwers, Wiqvist, Eriksson and Diczfalusy (1965) reported the identification of 15α-hydroxyestradiol-17β as a product of fetal liver metabolism. These workers administered estrone and/or estradiol-17β in the free as well as the sulfurylated form in vivo.

4. IDENTIFICATION OF ESTROGENS AS IN VITRO METABOLITES

The conversion of estrone to estradiol-17β and estriol as well as the conversion of estradiol-17β to estrone and estriol has been demonstrated in vitro by several groups of workers.

The first evidence for the presence of 17-epiestriol and 16,17-epiestriol was put forth by Breuer, Nocke and Knuppen (1958) and Breuer, Knuppen and Nocke (1959). They incubated human liver slices with 16α-hydroxyestrone and 16β-hydroxyestrone. The former yielded estriol, as the major conversion product, with small amounts of 17-epiestriol. The latter yielded 16-epiestriol as major conversion product with some 16,17-epiestriol. Breuer et al. (1958) also demonstrated that 16-ketoestradiol-17β was reduced to estriol and 16-epiestriol in vitro by human liver slices. Breuer and Nocke (1959) incubated 16-ketoestradiol-17α and were able to identify 17-epiestriol and 16,17-epiestriol. It should be emphasised here that 16-ketoestradiol-17α has not been identified in human urine.

Breuer, Knuppen and Pangels, (1959a), incubated human liver slices with 16-ketoestrone and identified the following compounds: 16α-hydroxyestrone, 16β-hydroxyestrone, 16-ketoestradiol-17β and the four estriol epimers. More recently, Lehmann and Breuer (1968) have studied the metabolism of 16-ketoestrone with various cellular fractions from the human placenta. Incubations of 16-ketoestrone with the 10,000 g
supernatant gave 16-ketoestradiol-17β and 16-epiestriol, whereas similar incubations with the cytoplasmic fractions produced 16α-hydroxyestrone, 16β-hydroxyestrone, estriol, 16-epiestriol. The 16-hydroxy compounds were rapidly reduced to estriol and 16-epiestriol. Incubations of 16-ketoestrone with microsomal fraction gave 16-ketoestrone, estriol and 16-epiestriol. These results demonstrated that the cytoplasmic fraction contained 16α-, 16β- and 17β-hydroxysteroid oxidoreductases of which the 17β-hydroxysteroid oxidoreductase showed the greatest activity. The microsomal fraction contained the 17β- and 16β-hydroxysteroid oxidoreductases only, suggesting pronounced differences in the enzymic activity and substrate specificity between the cytoplasmic and microsomal 17β-hydroxysteroid oxidoreductases.

Breuer, Knuppen and Haupt (1966) incubated liver slices with estrone and estradiol-17β. They isolated and identified the following compounds with estrone as the precursor: 6α-hydroxyestriol, estriol, 16-epiestriol, 7α-hydroxy estradiol-17β, 16α-hydroxy estrone, estradiol-17β and 2-methoxy estrone. With estradiol-17β as precursor the following were isolated and identified: 6α-hydroxy estriol, 6α-hydroxy estradiol-17β, 15α-hydroxy estradiol-17β, estriol, 16α-hydroxyestrone and estrone.

Knuppen, Haupt and Breuer (1965b) have incubated human adrenal tissue with estrone and have identified 15α-hydroxyestradiol-17β and 18-hydroxyestrone. A considerable amount of in vitro work has been done in connection with the metabolism of the C-2 substituted estrogens. Troen (1961) perfused human placentas with radioactive estradiol-17β in presence and in the absence of human chorionic gonadotrophin (HCG) and was able to detect
2-methoxyestrone. Lucis (1965) incubated human genital tract tissues with labelled methionine (methionine-methyl-\(^{14}\)C) and unlabelled estradiol-17\(\beta\) and was able to demonstrate the conversion of estradiol-17\(\beta\) to 2-methoxyestrone and 2-methoxyestradiol-17\(\beta\). Breuer, Pangels and Knuppen (1961) have demonstrated the conversion of 2-hydroxyestradiol-17\(\beta\) to 2-methoxyestradiol-17\(\beta\) \textit{in vitro} with human liver slices. The demethylation reaction has also been demonstrated by Mittermayer and Breuer (1962) and Breuer, Knuppen, Gross and Mittermayer (1964) \textit{in vitro}.

Gual, Morato, Hayano, Gut and Dorfman (1962) demonstrated the conversion of 11\(\alpha\)-hydroxy androst-4-ene-3, 17-dione to 11\(\alpha\)-hydroxy estrone by human placental microsomes. Ryan (1961) found that 11\(\alpha\)-hydroxy testosterone served as a precursor for 11\(\alpha\)-hydroxy estrone. Cedard and Knuppen (1965) have demonstrated 6\(\alpha\)-hydroxylation in human placenta by perfusion of testosterone-4-\(^{14}\)C; they identified 6\(\alpha\)-hydroxyestradiol-17\(\beta\). Lehmann and Breuer (1967a) have characterized and determined the kinetics of a 17\(\beta\)-hydroxysteroid oxidoreductase in the human placenta. Slaunwhite, Karsay, Hollmer, Sandberg and Niswander (1965) found that tissues of placentas from the 3rd – 4th month of gestation were capable of hydroxylating estrone both in 16\(\alpha\)- and 16\(\beta\)- position, whereas those from the 9th month of gestation were inactive.

Starka, Janata and Novak (1966) incubated placental tissue culture and corpus luteum tissue culture (60th day of gestation) with 7\(\alpha\)-hydroxy-dehydroepiandrosterone and its 3-sulfate. They identified 7\(\alpha\)-hydroxyestradiol-17\(\beta\) and 7-ketoestradiol-17\(\beta\) from the placental incubations and 7\(\alpha\)-hydroxyestrone, 7\(\alpha\)-hydroxyestradiol-17\(\beta\) and 7-ketoestradiol-17\(\beta\) from the corpus luteum incubations. Similar results were obtained by Cedard,
Fillmann, Knuppen, Lisboa and Breuer (1964). These results demonstrated that both placenta as well as the corpus luteum can be a source of 7-oxygenated estrogens, but C-7 substituted estrogens have not been identified as in vivo conversion products to date.

Flickinger, Wu and Touchstone (1967) obtained a 33-44% conversion of dehydroepiandrosterone-7α-3H to estrone and estradiol-17β by corpora lutea of early human pregnancy. Interestingly enough, the major conversion product was estrone when tissue homogenate was incubated, whereas estradiol-17β was the major conversion product when tissue slices were incubated. Wu, Flickinger, and Touchstone (1967) have extended the above work to placental, corpus luteal and fetal visceral tissues and have demonstrated that DHA-7α-3H is converted by these tissues to estrone and estradiol-17β, thereby demonstrating that DHA may serve as a precursor for estrogen biosynthesis not only in the feto-placental compartment but also in the corpus luteum. Ring B unsaturated compounds have not been identified in human species as in vivo conversion products. However, Starka, Breuer and Cedard (1966) have reported the formation of equilin, equilenin, dihydroequilin-17β and dihydroequilenin-17β following the perfusion of androsta-4,7-diene-3,17-dione, 3β-hydroxyandrost-5,7-dien-17-one and 3β,7α-dihydroxyandrost-5-en-17-one through a full term placenta, 20-30 minutes after delivery. The only compound, of the ones used for the perfusion experiment, known to occur as a metabolite in man, is 3β,7α-dihydroxyandrost-5-en-17-one.

In the last five years a considerable amount of information has been accumulated about the biosynthetic and conjugation reactions occurring in the feto-placental compartment and the implication of the fetal adrenal
and fetal liver as active biosynthetic tissue in the high production of estrogens during pregnancy. These experiments have utilized the techniques of in situ perfusion of labelled steroid precursors and their conjugates and in vitro incubations with precursors. A review of this work is outside the scope of this thesis, therefore references are made to important reviews and reports by various groups of workers. Baulieu et al. (1965) have made an extensive review of the production of dehydroepiandrosterone and dehydroepiandrosterone sulfate in the fetal adrenals and their conversion into estrogens. More recently, Johanniesson (1968) has studied the fetal adrenal cortex, its ultrastructure at different stages of development and its different functional states. Diczfalusy and Benagiano (1966) have reviewed the metabolism of estrogens in the feto-placental unit at mid-term. The transport of estriol and its conjugates across the placenta has been reviewed by Levitz et al. (1967).

5. METABOLISM OF ESTROGENS IN THE HUMAN

The state of knowledge regarding the metabolism of estrogens up until about 1950 could be summarized in a scheme given by Pincus and Pearlman (1943) as follows:

\[
\text{estradiol-17β} \leftrightarrow \text{estrone} \rightarrow \text{estriol} \downarrow \text{estradiol-17α (in other mammals)}
\]

Figure I: Metabolic scheme of estrogens by Pincus and Pearlman (1943)
Nothing was known as to how the diols were converted to estriol. It was believed that conversion of estradiol-17β to estrone was an obligatory step in the conversion of the diol to the triol, but there was no direct evidence either to prove or disprove this theory. Furthermore, there was no evidence to show if the conversion of estradiol-17β to estradiol-17α in the rabbit was merely an epimerization of the 17α-hydroxyl group or whether dehydrogenation to estrone was an obligatory step.

There was some information regarding the degree of interconversion of the classical estrogens mainly obtained through study of estrogen metabolites labelled with $^{131}$I in ring A (Heard and Saffran, 1949). There was no new information regarding estrogen metabolism.

Heard (1949) modified the scheme of Pincus and Pearlman to take into account estrogens identified by partition methods also. His scheme is as follows:

\[ \text{estradiol-17β} \xrightarrow{\text{reactions}} \text{estradiol-17α (in other mammals)} \]

\[ \text{estradiol-17α} \xrightarrow{\text{reactions}} \text{estrone} \]

\[ \text{estrone} \xrightarrow{\text{reactions}} \text{estriol} \]

\[ \text{estriol} \xleftarrow{\text{reactions}} \text{estradiol-17β} \]

\[ \text{estradiol-17α} \xleftarrow{\text{reactions}} \text{estrone} \]

![Figure II: Metabolic scheme of estrogens by Heard (1949).](image)

The solid arrows in the above diagram indicate reactions established by isolation of the crystalline compound, the dotted arrows indicate reactions established by partition techniques alone, while crossed solid arrows indicate reactions which could not be demonstrated. It was believed that reactions leading to the formation of estriol were
irreversible and there was even a belief that estriol formation was a characteristic of the human. The latter belief was shown to be entirely incorrect by the discovery of estriol in other vertebrates. Heard's scheme basically still holds true, but the metabolic scheme for estrogens is now more complex because of the numerous metabolites which have since been discovered.

Significant contributions were made by Fishman's group in the study of the kinetics of the interconversion of estrone and estradiol-17β. Prior to their work, Beer and Gallagher (1955a,b) showed that when estrone and estradiol-17β were given in tracer doses, estriol was the main metabolite in the urine of the first day of the experiment; in contrast, when large doses of estradiol-17β were administered, estrone was the principal metabolite during and immediately following either the injection or feeding of estradiol-17β. Brown (1957) reinvestigated the urinary excretion of estrone, estradiol-17β and estriol following an intramuscular injection of each of these three estrogens. He found that approximately equal amounts of estriol were formed from estrone and estradiol-17β, suggesting that a equilibrium between estrone and estradiol-17β was attained very rapidly.

Fishman, Bradlow and Gallagher (1960) administered estrone-16-14C and estradiol-17β labelled with tritium in ring B simultaneously into three subjects. These studies showed that urinary estrone and conjugated blood estrone, when measured 15 minutes after injection, had virtually the same isotopic ratio as the injection mixture; in contrast, the urinary estradiol-17β and the conjugated blood estradiol-17β contained very little 14C radioactivity. The only means by which tritium counts could appear
in estrone was the oxidation of estradiol-17β \textit{in vivo}. The $^{14}\text{C}$ to $^3\text{H}$ ratios suggested that estradiol-17β was very rapidly converted to estrone. These results suggested that estrone rather than estradiol-17β was the precursor of estriol. Langer and Engel (1958) have isolated and purified an enzyme from human placenta which catalyzes the estrone-estradiol-17β interconversions and is known as estradiol-17β dehydrogenase. This enzyme requires NAD or NADP as a coenzyme and has been shown to be specific for the 17β-hydroxyl group. Numerous studies describing the properties of this enzyme have been reported in literature.

Further experiments were done by Fishman, Bradlow, Hellman and Gallagher (1961) with the aim of determining the true precursor of estriol. They injected estradiol-17β labelled with tritium in the 17α-position into human subjects. Most of the radioactivity appeared in the body water. Moreover, the estriol fraction did not have any radioactivity. This experiment demonstrated that estrone was an immediate precursor of estriol. However, there is also some \textit{in vitro} evidence which suggests that the liver and other organs are capable of converting estradiol-17β to estriol directly. Quantitatively, however, the 16-hydroxylation of estradiol-17β is insignificant, at least in the human.

The subsequent steps by which estriol and 16-epiestriol are derived have been the subject of considerable speculation particularly with respect to 16-keto compounds, 16-ketoestradiol-17β and 16-ketoestrone. It has been suggested that these compounds are either the precursors or oxidation products of estriols in humans. Following the discovery of 16-epiestriol and 16α-hydroxyestrone, Marrian et al. (1957) proposed that 16α-hydroxyestrone and 16β-hydroxyestrone were intermediates in the formation
of estriol and 16-epiestriol, respectively, and were formed by 16α- or 16β-hydroxylation of estrone. This hypothesis received support from the *in vivo* and *in vitro* evidence presented by Breuer and his group (Breuer *et al.*, 1958; Nocke *et al.*, 1961).

The experiments by Fishman *et al.* (1961) showing that estrone rather than estradiol-17β was the preferred precursor of estriol have been described above. They suggested that 16α-hydroxylation of a 17-ketone occurred more readily than 16α-hydroxylation of a 17β-hydroxy compound, at least in the human. In keeping with this hypothesis, Hobkirk (1963) detected radioactivity in the 16α-hydroxyestrone fraction from the urine after injection of estrone-16-14C into non-pregnant subjects. However, no conclusive evidence for the formation of 16β-hydroxyestrone from estrone has been forthcoming. It was theorized, therefore, that in the formation of 16-epiestriol from estrone, introduction of the hydroxyl group in the 16β-hydroxy position did not occur directly but resulted from the reduction of a 16-keto group. A compound eminently suitable to play a key role was 16-ketoestrone, which, according to Huffman and Grollman (1947), was formed directly from estrone. Such a key role for 16-ketoestrone had already been proposed by Breuer (1962). The conversion of 16-ketoestrone to estriol, 16-epiestriol and 16-ketoestradiol-17β (Stimmel *et al.*, 1948, 1950; Stimmel, 1958) supported this hypothesis. The conversion of estrone to estriol, 16-epiestriol and 16-ketoesradiol-17β *in vivo* has already been demonstrated by Hobkirk, 1963; Migeon *et al.*, 1959; Fishman *et al.*, 1960; Hobkirk *et al.*, 1966; and Hobkirk and Nilsen, 1966.
The second 16-keto compound 16-ketoestradiol-17β was also believed to play an important role in the formation of the estriol epimers, since 16-ketoestradiol-17β was reduced to estriol and 16-epiestriol \textit{in vivo} as well as \textit{in vitro} (Beling \textit{et al.}, 1963; Stimmel \textit{et al.}, 1950; and Breuer \textit{et al.}, 1958). The work by Levitz \textit{et al.} (1958) and Fishman \textit{et al.} (1968) clearly demonstrated that 16-ketoestradiol-17β arises by the partial oxidation of estriol but without the formation of a 17-keto compound as an intermediate. This would suggest that 16-ketoestradiol-17β was an oxidation product of estriol. The interconversion of estriol and 16-epiestriol could be explained by oxidoreduction via 16-ketoestradiol-17β.

Recently, Fishman, Hellman, Zumoff and Cassouto (1966) have studied the formation of estriol in man with the aim of elucidating the role of the 16-keto compounds as key intermediates in the estriol pathway. The stereochemical nature of the C-16 hydroxylation has been of particular interest since estrone is the substrate involved in the 16-hydroxylation, therefore, an enolizable hydrogen α to a ketone is involved. It had been demonstrated earlier (Hayano, Gut, Dorfman, Sebek and Peterson, 1958) that enzymatic hydroxylation at other unactivated positions in the steroid molecules proceeded with a replacement reaction rather than a displacement reaction. This need not be the case with hydroxylations at carbon where an enol may be involved. Fishman \textit{et al.} (1966) injected estradiol-17β-16α-^{3}H and estradiol-17β-16β-^{3}H separately into human subjects and determined the tritium content of urinary estriol and 16-epiestriol. No tritium label was found in the estriol fraction when estradiol-17β-16β-^{3}H was injected but the label was retained in the corresponding 16-epiestriol fraction. On the
other hand, no tritium label was found in the 16-epiestriol fraction when estradiol-17β-16α-3H was injected, however the label was retained in the corresponding estriol fraction. These results suggest that hydroxylation at C-16 position, both in the 16α- as well as the 16β-position, proceed by a replacement of hydrogen and that no common enol form is involved. Furthermore, these results suggest that the 16-keto compounds cannot be regarded as intermediates in the biosynthesis of estriol and 16-epiestriol.

These results conclusively show that the earlier hypotheses regarding the key role of the 16-keto compounds in the biosynthesis of estriol and 16-epiestriol were incorrect. The formation of 16-keto compounds, mainly, 16-ketoestradiol-17β which has been shown to be definitely present, could be due to the oxidation of estriol. Recently Dahm, Lindlau and Breuer (1968) have characterized a steroidepimerase in the human placenta which converts 16α-hydroxyestrone to 16β-hydroxyestrone and vice versa and does not require the formation of 16-ketoestradiol-17β as an intermediate. These results essentially support the findings by Fishman et al. (1966), in so far as they strongly suggest that 16β-hydroxyestrone which is the immediate precursor of 16-epiestriol in humans, can arise from 16α-hydroxyestrone without formation of 16-keto compounds. The various reactions which the C-16 substituted phenolic steroids undergo are basically 6 reactions that involve the following enzymes: 16α-, 16β-hydroxylases; 16α-, 16β-, 17β- and 17α-hydroxysteroid oxidoreductases.

The presence of 17-epiestriol and 16,17-epiestriol in the urine (Breuer and Pangels, 1961) and the formation of these estriols in vitro
by human liver slices from 16α-hydroxyestrone and 16β-hydroxyestrone (Breuer et al. 1958; Breuer, Knuppen and Nocke, 1959) showed that 17α-hydroxysteroid dehydrogenase was present in humans. In view of these findings, it is surprising that estradiol-17α has not been isolated from human pregnancy urine, although Schott and Katzman (1964) identified it in the urine of one person who had a history of endocrine disorder. These workers suggested that the failure to identify estradiol-17α normally may be due to its rapid metabolic alteration. Breuer and Schott (1966) administered estradiol-17α orally to healthy subjects to study the fate of estradiol-17α. Increases in the excretion of estrone, estradiol-17β and estriol fractions in urine were observed. After processing the estrone fraction, estrone was obtained in crystalline form. This result leaves no doubt that 17α-hydroxysteroid dehydrogenase which has been shown to be present in humans reacts with estradiol-17α. Breuer and Schott (1966) also showed that estradiol-17α, in contrast to estradiol-17β, is metabolized very rapidly in vivo since only 3.5% of the given dose was recovered in estradiol-17β, estrone and estriol-like fractions, whereas when estradiol-17β was administered under similar experimental conditions, 20% of the dose was recovered from the urine in the estrone, estradiol-17β and estriol fractions. The in vivo results with estradiol-17α were confirmed by in vitro experiments. On the basis of these findings the authors have suggested that the difficulties in isolating estradiol-17α from human urine are due to, first, presence of very small amounts of estradiol-17α and second, the very rapid metabolism of estradiol-17α formed.

More recently, Williams and Layne (1967) studied the metabolism of estradiol-17α in non-pregnant women. Estradiol-17α-6,7-3H was administered
intravenously and approximately 58-65% of the injected radioactivity was recovered in the urine over a period of four days. Over 90% of the material conjugated with glucuronic acid was shown to be unchanged estradiol-17α. The major metabolite of estradiol-17α was identified as 2-methoxyestradiol-17α. In contrast to the findings by Breuer and Schott (1966), Williams and Layne (1967) were unable to obtain any evidence for the conversion of estradiol-17α to estrone. They have suggested that the results obtained by Breuer and Schott (1966) could be explained on the basis that their large doses of estradiol-17α were converted to very small amounts of estrone and polar metabolites; and they have also suggested that the 17α-hydroxysteroid dehydrogenase activity is not extensive in humans and that the presence of a 17α-hydroxyl group almost completely inhibits the metabolism of ring D.

The metabolism of C-2 substituted estrogens has been investigated in detail. Studies on the effect of thyroid function on the formation of the 2-hydroxy estrogens and 2-methoxy estrogens indicate that C-2 hydroxylation is accentuated at the expense of C-16 hydroxylation when thyroid activity is relatively high, and vice versa (Fishman, Hellman, Zumoff and Gallagher, 1965). Brown and Strong (1965), on the other hand, have provided evidence which suggests that the metabolism of estrogens is related to the body weight of an individual rather than the thyroid function. It is now well established that 2-methoxyestrogens arise from the methylation of the corresponding 2-hydroxy compounds; furthermore, 2-hydroxylation and methylation play an important part in the intermediary metabolism of estrogens. The demonstration that demethylation occurs in vitro and in vivo (Mittermayer and Breuer, 1962; Hobkirk
and Nilsen, 1963; Breuer, Knuppen, Gross and Mittermayer, 1964) suggests that the 2-hydroxy and 2-methoxy estrogens may have regulatory functions in the intermediary metabolism of estrogens (Breuer, 1964).

The metabolism of the C-15 substituted estrogens is of great interest, since these compounds have been discovered only in the last three years from human sources. The present studies indicate the presence of both the 15α- and 15β- hydroxysteroid dehydrogenases in the human liver (Knuppen and Breuer, 1966).

So far there is very little information on the metabolism of the C-6 and C-7 substituted estrogens in humans. In vivo and in vitro studies (Breuer and Breuer 1965; Cedard and Knuppen, 1965) have shown that 6α-, 6β-, hydroxysteroid dehydrogenases are present in humans. Both 7α- and 7β- hydroxysteroid dehydrogenases are present in the placenta and the corpus luteum since 7α- hydroxy dehydroepiandrosterone which is a normal steroid metabolite in man (Starka, Sulcova and Silink, 1962) can be aromatized to 7α-hydroxyestrone (Cedard et al., 1964) and to 7-ketoestradiol-17β and 7-hydroxyestradiol-17β (Starka et al., 1966).

The intermediary metabolism of estrogens in humans based on the in vivo and in vitro evidence presented in the preceding two sections is summarised in Figure III.
Figure III (continued)

Intermediate Metabolism of C-2, C-6, C-15 and C-18 Substituted Estrogens in Humans
B. OCCURRENCE AND METABOLISM OF ESTROGENS IN CERTAIN MAMMALIAN SPECIES OTHER THAN HUMANS

1. INTRODUCTION

Domestic animals and experimental animals, such as rats, mice and guinea pigs, have been used extensively in investigation of the physiological effects of estrogens and in the elucidation of the pituitary-ovary relationship. Apart from these studies, others have been done to ascertain the nature and amounts of the estrogens excreted by various animals. The earlier studies were, for the most part, based on bioassay techniques for identification of steroidal estrogens. Bioassay techniques permitted detection of the active forms of estrogens alone, whereas estrogens are excreted in both active and relatively inactive forms. Detection of the inactive forms of estrogens necessitated the use of chemical methods. The material presented in this section is primarily based on identifications by chemical methods.

Of the mammals studied, the horse aroused much interest because the ring B unsaturated steroidal estrogens were found to occur only in the equine species. The horse was also shown to possess the most complex metabolic pathway for estrogens amongst the various mammals studied. A more intensive study of estrogen metabolism in various mammalian species has been done over the last fifteen years using radioactive tracers both in in vivo and in vitro experiments.

2. ISOLATIONS

All the tissues which have been shown to produce estrogens in the human also produce estrogens in various mammals. Estradiol-17β and
estrone have been detected in most of the hormone-producing tissues of all the mammals studied. The placenta of the cow produces estradiol-17α along with estrone and estradiol-17β, whereas goat and sheep placentas have been shown to produce estradiol-17α alone. Interestingly enough, the testis of the stallion and the boar produce considerable amounts of estrone and estradiol-17β. A high production of estrogens by the testis has not been demonstrated in other mammalian species studied so far. Table III summarizes the estrogens identified in the hormone producing tissues of certain mammalian species.

The urine of these mammals has also been studied, more especially the pregnancy urine. Again, estrone and estradiol-17β have been identified in the urine of all the mammals studied so far with the exception of the sheep and the goat, in which species estrone has been identified.

Klyne and Wright (1957, 1959) subjected the urine from cows and goats to acid hydrolysis to liberate phenolic steroids and obtained higher values for estrone than for estradiol-17α. It is well known that acid hydrolysis causes almost complete destruction of estradiol-17α, consequently the relative values of estrone and estradiol-17α obtained by Klyne and Wright could not be taken to represent the actual quantitative situation. Velle (1958a) used enzymatic hydrolysis for liberation of phenolic steroids and demonstrated that the values of estradiol-17α were consistently several times greater than the corresponding values for estrone. Estradiol-17α rather than estrone is, therefore, the major urinary estrogen in the cow and the goat. Estradiol-17α represents the major urinary estrogen in the ruminants, and this indicates that this
TABLE III.

Steroid estrogens isolated and identified in the hormone producing tissue of certain mammalian species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Source</th>
<th>Estrogen</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stallion</td>
<td>Testes</td>
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<td>Beall (1940)</td>
</tr>
<tr>
<td>Mare</td>
<td>Follicular fluid</td>
<td>Estrone</td>
<td>Short (1960)</td>
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<td></td>
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<td>Estradiol-17β</td>
<td>&quot; &quot;</td>
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<tr>
<td></td>
<td></td>
<td>6α-hydroxy-</td>
<td>Bush, Klyne &amp; Short (1960)</td>
</tr>
<tr>
<td></td>
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<td>estradiol-17β</td>
<td></td>
</tr>
<tr>
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<td>Follicular fluid</td>
<td>Estradiol-17β</td>
<td>MacCorquodale, Thayer &amp; Doisy (1936)</td>
</tr>
<tr>
<td></td>
<td>Ovaries</td>
<td>Estrone</td>
<td>Westerfeld, Thayer</td>
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<td></td>
<td>MacCorquodale &amp; Doisy (1938)</td>
</tr>
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<td>Placenta</td>
<td>Estrone</td>
<td>Velle (1958a)</td>
</tr>
<tr>
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<td>Testes</td>
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<td>Velle (1958a)</td>
</tr>
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<td>&quot; &quot;</td>
</tr>
<tr>
<td>Cow</td>
<td>Follicular fluid</td>
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<td>&quot; &quot;</td>
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</tr>
<tr>
<td>Ox</td>
<td>Adrenal</td>
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<td>Sheep</td>
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<td>Velle (1958a)</td>
</tr>
<tr>
<td>Goat</td>
<td>Placenta</td>
<td>Estradiol-17α</td>
<td>Velle (1963)</td>
</tr>
</tbody>
</table>

* denotes tentative identification.
compound represents the end product of estrogen metabolism in these mammals just as estriol represents the end product of estrogen metabolism in the human.

The levels of steroid estrogens in stallion urine and boar urine have been determined. The estrogen levels for the stallion are of the order of 20 mg. estrone and 2 mg. each of estradiol-17α and estradiol-17β per litre of urine (Pigon, Lunaas and Velle 1961). The estrogen levels in the adult, fertile boar have been found to be 2 mg. of estrone and 1 mg. of estradiol-17β per litre of urine (Velle 1958b). Such high values for estrogens in the male urine have not been recorded for any other mammalian species studied so far. Table IV summarizes the estrogens isolated and identified from the urines of certain mammalian species.

In many mammalian species, the urine is not the only route by which estrogens are excreted. Pearlman, Rakoff, Cantarow and Paschikis (1947) examined the bile from cows for estrogens and concluded that estrone was the major estrogen in the bile and that most of the estrogenic material was present in the free form. The evident importance of biliary excretion of estrogens would suggest that fecal excretion of estrogens should also take place. The study of fecal material presents certain difficulties due to the presence other lipid materials. Levin (1945b), however, made a systematic study of the feces from cows at various stages of pregnancy. He found that the major portion of the estrogenic activity was concentrated in the non-ketonic fraction separated in the course of partition studies. The activity in this fraction was 5000–10,000 rat units of estrogenic substance. This activity calculated as estradiol-17β was equivalent to 0.9–1.4 mg per kilogram of feces. Levin attributed the predominance of non-ketonic
Steroid estrogens isolated and identified from the urine of certain mammalian species other than humans.

<table>
<thead>
<tr>
<th>Species</th>
<th>Estrogens</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Rhesus monkey</td>
<td>Estrone Estradiol-17β</td>
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<tr>
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<td>(female)</td>
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<td></td>
<td>Estriol*</td>
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</tr>
<tr>
<td>Cow</td>
<td>Estrone Estradiol-17β 16,17-epiestriol</td>
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<td>(pregnancy)</td>
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<td>Mellin, Erb &amp; Estergreen (1965)</td>
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<td>(pregnancy)</td>
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<td>Estriol</td>
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</table>

* denotes tentative identification
estrogen material in the feces of the cow to reduction by intestinal bacteria. The mare, on the other hand, does not excrete appreciable amounts of estrogens via the bowels (Solomon and Heard, 1952). Wright (1962) has shown that the feces constitute the major excretory route for estrogens in the sheep. The species difference in the route of excretion of estrogens possibly reflects a difference in the metabolism of the steroidal estrogens.

3. IDENTIFICATION OF ESTROGENS AS IN VIVO METABOLITES

The interconversions of the 'classical' estrogens were studied in certain mammalian species concurrently with similar studies made in the human. Administration of exogenous estrogens and subsequent identification of conversion products was a standard approach in the investigation of the metabolism of estrogens.

Westerfeld and Doisy (1937) administered estrone to an ovariectomized and hysterectomized monkey and obtained evidence for the presence of non-ketonic estrogens. Dorfman, Wise and Van Wagenen (1945) isolated estrone from the urine of ovariectomized—hysterectomized rhesus monkeys which had received either estradiol-17β-dipropionate or estrone propionate. These experiments demonstrated the interconversion of estrone and estradiol-17β in the rhesus monkey. The interconversion of estriol in this species was studied by Doisy, Thayer and Van Bruggen (1942). They injected estriol into normal intact monkeys as well as ovariectomized—hysterectomized monkeys and were unable to detect any increase in the ketonic fraction or the estradiol-17β fraction. These experiments, furthermore, demonstrated very clearly that the uterus and the ovaries were not essential for the inter-
conversion of estrogens. Later work with liver slices showed that the interconversion of estrogens was effected by the peripheral tissues.

The metabolism of estrogens in the rhesus monkey has been investigated more recently by Flickinger and Wu (1967) using labelled estrogen. They injected estradiol-17β-4-14C into a non-pregnant monkey. Estrone and estradiol-17β were found to be the major urinary radioactive metabolites. Small amounts of estriol and 16-epiestriol were also detected.

Jirku and Layne (1965) injected a tracer dose of estrone-16-14C into a pregnant chimpanzee and identified the following radioactive steroids from the urine: estradiol-17β, estriol and 2-methoxyestrone. The specific activities of the estrone and estradiol-17β were 100 times that of estriol, although the latter estrogen was present in large amounts. This suggests that a major part of the urinary estriol had been formed by a route which did not involve the reduction of estrone. These results are very similar to those obtained with the human, but there is one important difference, to wit, there is practically no free estrogen in the human, whereas in the chimpanzee half the injected dose is excreted as unconjugated steroid.

To the best of the author's knowledge, there have been no other reports about the metabolism of estrogens in the primates.

The rabbit has been a subject of investigation for a long time. Stroud (1939) isolated approximately 6% estradiol-17α from normal rabbits treated with estrone. He was unable to detect estradiol-17β. These observations were further confirmed by Fish and Dorfman (1942). They administered estradiol-17β to ovariectomized-hysterectomized rabbits and identified estrone and estradiol-17α. Heard, Bauld and Hoffman (1941) obtained similar results with intact rabbits in estrus as well as
ovariectomized-hysterectomized rabbits. They injected either estrone or estradiol-17β with or without simultaneous treatment with progesterone and found estradiol-17α to be the major urinary estrogen, while estrone was also detected. Pincus and Zahl (1937) and Heard, Bauld and Hoffman (1941) could not demonstrate the conversion of estriol to any other estrogen in the rabbit. They were able to detect only unchanged estriol in the urine.

In the last five years, the metabolism of estrogens in the rabbit has been studied in connection with the identification of the estrogen conjugate excreted by the rabbit. Layne, Sheth and Kirdani (1964) administered estrone-16-14C to New Zealand white rabbits and isolated a diconjugate of estradiol-17α which was shown to be conjugated with N-acetyl glucosamine in the C-17 position and glucuronic acid in the C-3 position (Layne 1965).

So far the rabbit is the only species in which estrogens have been shown to be conjugated with N-acetyl glucosamine. More recently, Williams, Henry, Collins and Layne (1968) have studied the metabolism of 4-14C-17β-3H-estradiol-17α and 16-14C-17α-3H-estradiol-17β in rabbits to determine if the conversion of estradiol-17β to estradiol-17α was the result of epimerization of the hydroxyl group in the C-17 position. When the former compound was injected the tritium label was completely retained by the urinary estradiol-17α, moreover the injected estradiol-17α did not give rise to any other radioactive metabolites, but was conjugated and excreted as such. When the latter compound was injected, there was complete loss of tritium in the urinary estradiol-17α. These experiments provided an elegant demonstration that estrone formation was an obligatory step in the conversion of estradiol-17β to estradiol-17α and, furthermore, that estradiol-17α represents the end product in the metabolism of estrogens in the rabbit.
The metabolism of estrone-16-\(^{14}\)C has been investigated in other rodents, such as mice, rats and the golden hamster. No positive identifications were made in the former two rodents. Budy (1955) found radioactivity in the estrone and estradiol-17\(\beta\) fractions in the urine of mice after injection of labelled estrone. Valcourt, Thayer, Doisy, Elliot and Doisy (1955) observed radioactivity in the phenolic and neutral fractions after injection of estrone-16-\(^{14}\)C into rats. More recently Collins, Williams and Layne (1967) have studied the metabolism of radioactive estrone and estradiol-17\(\beta\) in the golden hamster. They found that the urinary metabolites were the same whichever of the two steroids was administered; and Collins et al. (1967) identified estrone, 2-methoxyestrone and 2-hydroxyestrone rigorously. Quantitatively, 2-hydroxyestrone was the major radioactive metabolite. There was some evidence for the presence of 2-hydroxyestradiol-17\(\beta\). The golden hamster appears, therefore, to metabolize ring A effectively.

In the earlier studies on the metabolism of estrogens in the dog, bioassay techniques were used for the identification of estrogens, hence the work was limited in scope. However, Pearlman, Rakoff, Paschikis, Cantarow and Walkling (1948) identified estradiol-17\(\beta\) in the bile following subcutaneous injection of estrone sulfate using bioassay techniques. Mayer (1952) identified estrone, estradiol-17\(\beta\) and estriol as urinary conversion products of estradiol-17\(\beta\). However, the analytical methods used in the identification of estriol were not particularly specific, so that conclusive proof of the presence of estriol is still lacking. Siegal, Dorfman, Brodey and Friedman (1962) identified estrone estradiol-17\(\beta\) and estradiol-17\(\alpha\) in the urine of one dog following injection of estradiol-17\(\beta\)-
6,7-^H. Very small amounts of radioactivity that may have been due to estriol were found but this substance was not formally identified. Kristoffersen and Velle (1960), on the other hand, did not find any significant amounts of endogenous estrone, estradiol-17β or estriol in untreated male dogs or pregnant female dogs. These results conflict with the findings of Siegal and Dorfman (1963), who were able to identify endogenous estrone and estradiol-17β in the urines of two dogs studied. A more definitive study was undertaken by Balkian, Southerland, Howard and Preedy (1968) to resolve the conflicting results obtained by previous investigators. They administered estrone-6,7-^H to a group of male dogs and studied the uptake of radioactivity by various tissues. The tissues, plasma and urine were analysed for specific radioactive estrogens. Estrone and estradiol-17β were found in all tissues and in plasma and urine. A preferential concentration of estrone was observed in the omentum, whereas there was no similar concentration of estradiol-17β in any tissue. There was no evidence for the presence of 2-methoxyestrone in any tissue, plasma or urine. There was some evidence for the presence of estradiol-17α in the liver tissues but the identification was incomplete. Small amount of 17-epiestriol was identified in the liver and kidneys. Relatively high amounts of 17-epiestriol were found in all the urine samples, whereas traces of estriol were identified in only half the urine samples analyzed. These experiments showed that 17-epiestriol was the major estriol epimer present in the dog urine.

There is relatively little information concerning the metabolism of estrogens in vivo in the ruminants. Velle (1958d) administered unlabelled estradiol-17β to young calves and found estrone and estradiol-17β in the
Hunt, Legault and Herrick (1961) essentially confirmed these findings by the administration of estrone-16-$^{14}$C and identification of labelled estrone, estradiol-17α and 16-epiestriol in the urine. Velle (1958e) also demonstrated that estradiol-17α was converted to estrone by young calves.

There has been one report so far concerning the metabolism of estrogens in vivo in the pigs. Lunaas (1963) demonstrated the conversion of estradiol-17β to estrone in the pig.

4. IDENTIFICATION OF ESTROGENS AS IN VITRO METABOLITES

In vitro studies have been carried out, primarily, with the aims of localizing the sites of metabolism of estrogens and of isolating the enzymes involved in the metabolism. Such a study has not been carried out on an extensive scale with various mammals but these limited in vitro studies have supported the view that marked species differences do exist. Much of the earlier investigations were based on the bioassay techniques and no definite conclusions could be drawn about the metabolites formed. However, the in vitro studies (Heller, 1940) with rat and rabbit liver, demonstrated conclusively the importance of the liver in carrying out estrogen transformations.

The tissues most frequently studied have been the liver, adrenals and kidneys. The incubations of these tissues has been done either as tissue slices, homogenates or microsomal fractions. Blood cells also have been used in studying the transformations of estrogens.

The conversion of estradiol-17β to estrone in vitro by liver slices has been demonstrated for the rat (Pearlman and DeMeio, 1949; Ryan and Engel,
1960) and the rabbit (Breuer and Pangels, 1960). The reverse re-
action, that is, the conversion of estrone to estradiol-17β has been
demonstrated in the rat liver (Ryan and Engel, 1953), bovine liver
(Erichsen and Velle, 1960) and rabbit liver (Breuer and Pangels, 1960).
Crepy and Jayle (1965) have investigated the in vitro conversion of
estrone sulfate by rabbit liver slices. After 45 minutes of incubation
only estradiol-17β and estradiol-17α were identified. However, after
3.5 hours of incubation, the following estrogens were identified on the
basis of chromatographic mobility:- estrone, 6-ketoestradiol-17β,
16-epiestriol, 16,17-epiestriol, and estradiol-17β. It should be noted
in this connection that Williams et al. (1968) were unable to demonstrate
the conversion of estradiol-17α to estrone in vivo in the rabbit.
Furthermore Stroud (1939) was unable to show the conversion of estrone to
estradiol-17β in vivo. In vitro results indicate, therefore, reactions
feasible under experimental conditions rather than the actual series of
transformations occurring in the intact animal. However, the value of
such experiments should not be underestimated since they demonstrate the
enzymes present in the tissue which bring about transformations in
estrogens. These enzymes may or may not be active in the intact animal.
Even in in vivo experiments, only those experiments which employ a true
tracer dose will reflect the reactions occurring in the normal animal.

The formation of C-2 substituted in vitro metabolites has been
observed by many investigators. King (1961a) identified 2-methoxy-
estradiol-17β as a conversion product of estradiol-17β when incubated
with a rat liver preparation. Similarly, estrone can also be methoxylated
in the position ortho to the phenolic group. King (1961b) was able to
recover 2-hydroxyestriol and 2-methoxyestriol in significant amounts when rat and rabbit liver preparations were incubated with estriol.

Pangels and Breuer (1962) have shown that estradiol-17β is converted to estriol by rat liver slices. These workers have located 16α-hydroxylase in the microsomal fraction of the liver and the adrenals and have shown that NADPH is required as a cofactor. Rat liver microsomal preparations have also been shown by King (1961a) to convert estriol to 16-ketoestradiol-17β.

In connection with the investigation of the key role of 16-ketoestrone and 16-ketoestradiol-17β, Breuer, Knuppen, and Pangels (1959b) incubated rabbit liver slices with 16-ketoestrone and other C-16 substituted steroid estrogens. 16-Ketoestrone gave rise to the following steroids: 16-ketoestradiol-17β, 16β-hydroxyestrone, 16α-hydroxyestrone, 16-epiestriol, 17-epiestriol and estriol. Some 16,17-epiestriol was also detected.

Lehmann and Breuer (1967b) have carried out the above work further with rat liver cell fractions. They incubated 16-ketoestrone independently with rat liver cytoplasm fraction and microsomal fraction in presence of NADH or NADPH. In presence of NADPH the main metabolic pathway in the cytoplasm fraction involved the formation of 16α-hydroxyestrone and 16-ketoestradiol-17β, which were reduced to estriol, whereas in the microsomal fraction the main metabolic pathway led to 16β-hydroxyestrone, which was then reduced to 16-epiestriol. The amounts of reduction products formed in the cytoplasm fraction were independent of the co-factor added, whereas in the microsomal fraction NADPH was much more effective as co-factor than NADH. King (1960), using rat kidney
homogenates, showed that estriol was converted to 16-ketoestradiol-17β and that 16-epiestriol was converted to 16-ketoestradiol-17β and estriol. The experiments described above tended to support the theory that 16-ketoestrone and 16-ketoestradiol-17β were key compounds in the formation of estriol epimer. However, other experiments described elsewhere in this thesis have shown conclusively that for humans at least this theory is essentially incorrect.

A group of estrogens first detected in in vitro experiments with rat and mouse liver preparations comprises the C-6 substituted estrogens. Rumney (1956) and Mueller and Rumney (1957) incubated estradiol-17β-16-14C with mouse and rat liver microsomes in presence of NADPH and obtained evidence for the formation of 6-hydroxyestradiol-17β, 6-hydroxyestrone and 6-ketoestradiol-17β. These observations were corroborated by Breuer, Nocke and Knuppen (1959) with rat liver slices. The actual isolation of 6-hydroxyestradiol-17β as a metabolite of estradiol-17β and 6-hydroxyestrone as a metabolite of estrone was done by Breuer, Nocke and Pangels (1960). Moreover, on the basis of the maxima of the time curves they concluded that the 6-hydroxylase of the rat liver attacks both estrone and estradiol-17β, whereas the 16α-hydroxylase acts only on estradiol-17β. The absolute configuration of the hydroxyl group in the C-6 position was not known at the time these studies were made. However, Breuer, Knuppen and Pangels (1962) prepared 6α- and 6β- hydroxyestrone from 6α- and 6β- hydroxyandrost-4-ene-3,17 dione (the configuration of these compounds has been well established) and thus were able to establish the configuration of the reference compounds. The experiments of Breuer et al. (1960) were repeated by Breuer et al. (1962), who were then able to show that both
6α-, and 6β-hydroxyestradiol-17β were formed when estradiol-17β was the precursor. Knuppen, Behm and Breuer (1964) have shown that rat liver slices convert estriol to 6-hydroxyestriol. The exact configuration of this compound is not known.

Other unusual estrogens have been detected as in vitro metabolites of estrogens with bovine adrenal tissue. The following estrogens were identified as the in vitro conversion products of estrone: 7α-hydroxyestrone (Knuppen, Haupt and Breuer, 1964), 15α-hydroxyestrone (Knuppen and Breuer, 1964), 11β-hydroxyestrone (Knuppen and Breuer, 1962) and 14α-hydroxyestrone (Knuppen, Haupt and Breuer, 1967). The identification of 7α-hydroxyestrone and 15α-hydroxyestrone were the first indications of a 7α- and a 15α-hydroxylating systems in mammals. Levy, Hood, Chao and Carlo (1965) perfused beef adrenals with estradiol-17β and were able to identify yet another C-15 substituted steroid, namely, 15α-hydroxyestradiol-17β. They also identified 16-epiestriol. Loke, Watson and Marrian (1957) incubated ox adrenal homogenates with estrone and were able to detect small amounts of a steroid which was tentatively identified as 18-hydroxyestrone on the basis of the chromatographic mobilities. This cannot be regarded as a formal identification of the metabolite, but if it does exist, then it is probably formed by the adreno-cortical enzymes.

A comparative study of the transformations of estrogens effected by washed blood cells of different mammals was done by Lunaas and Velle (1960). They demonstrated the interconversion of estrone and estradiol-17β in man, rhesus monkey, rat, mouse, guinea pig, goat, sheep, horse, dog, rabbit and hen. The interconversion of estrone and estradiol-17α has been
demonstrated also in cattle (Portius and Repke, 1960a; Axelrod and Werthessen, 1960). Although estradiol-17α is formed in vivo in many species, including the rabbit and goat, it is only with cattle erythrocytes that estrone is converted to estradiol-17α. Portius and Repke (1960b) have also shown that rat and bovine erythrocytes convert 16-ketoestradiol-17β to 16-epiestriol. Their observations with rat erythrocytes were corroborated by Trachewsky and Hobkirk (1963, 1964), who furthermore have shown that cat erythrocytes convert 16-ketoestradiol-17β primarily to estriol.

These experiments with blood cells underscore the need to carry out controlled experiments with tissues so as to eliminate the possibility of transformations occurring through the actions of blood enzymes, which are generally present in tissue preparations.

Although the foregoing section on the estrogens in the mammals presents an incomplete picture regarding the estrogens elaborated and their metabolism in individual species, it is quite evident that there are some basic similarities with the human species, in so far as estrone and/or estradiol-17β are elaborated in the ovaries of all the animals studied just as in humans. Estriol, which was considered to be a metabolite to be found exclusively in the human, has been shown to occur in many species although some of the earlier identifications of this metabolite in species other than the human need to be examined critically. The metabolism of estrogens in many species is probably as complicated as it is in the human. The major end product of estrogen metabolism in the ruminants as well as in the rabbit is estradiol-17α, which has not been identified as a normal urinary product in the human up until now.
Ring B unsaturated estrogens have not thus far been isolated from any normal species other than the pregnant mare.
C. OCCURRENCE AND METABOLISM OF ESTROGENS IN CERTAIN AVIAN SPECIES.

1. INTRODUCTION

The earliest recorded experiment performed with the domestic fowl in relation to endocrinological studies was done by John Hunter. Burrows (1949) has described his work in the following manner: "John Hunter (1794) in the course of experiments on grafting, discovered that if the spur of a young cock were transplanted into the leg of a young hen, the tissue took root but no spur grew, while the intact spur on the other leg of the donor cock developed as usual. Hunter then grafted the rudimentary spur of a young hen into a young cock and observed that the transplanted spur grew nearly as fast and to as large a size as the natural spur on the cock's other leg. These experiments he repeated several times, always with the same results."

Half a century later, Berthold (1849) was able to demonstrate by means of testicular grafts that an endocrine secretion was responsible for sexual maturation in cocks.

Since then the physiological effects of estrogens in the domestic fowl have been studied in relation to lipogenesis, calcium metabolism, phosphorus metabolism, skeletal changes, compositions of blood and liver, changes in endocrine glands, secondary sexual characteristics and broodiness. These studies have led to important contributions in fundamental endocrinology. The domestic fowl has proved to be an extremely useful experimental animal, since they have externally visible and measurable secondary sex characters (comb, wattle) which are extremely sensitive to hormonal changes. The physiological aspect has been reviewed by several

Although a great deal was known about the physiological effects of estrogens, knowledge which has been exploited commercially in improving the quality of chicken and turkey, nothing was known until relatively recently about the nature of phenolic steroids elaborated by birds themselves. Before any studies on the metabolism of estrogens of the fowl could be undertaken it was important to know the nature of the estrogens produced endogenously. Some knowledge has been forthcoming over the past decade on the occurrence and metabolism of estrogens in avian species. Most of the work has been done with the domestic fowl.

2. ISOLATIONS.

The earliest report on the detection of estrogens in the avian species was by Gustavson (1931) who detected estrogenic activity in the extracts from feces (mixed excreta?) when tested by bioassay. However, the first report of identification based on chemical methods was done by Hurst, Kuksis and Bendell (1957). These workers reported the presence of estrone, estradiol-17β and estriol in cock dropping and estrone and estriol in hen droppings. Their identifications were based on paper chromatography of extracts of the droppings and subsequent colour reactions. Their identification of estriol is open to criticism in view of the subsequent work on the identification of estrogens and the quantitative chemical estimations of the major urinary estrogens. The next report on identification of avian estrogens was by Layne, Common, Maw and Fraps (1958). They reported the identification of estrone and estradiol-17β
and, with lesser certainty, of estriol in extracts of the ovaries of laying hens. However, it is now known that the systems used for chromatographic separation of the ovarian extract (Layne, 1957) would not have effected a separation of the estriol epimers and the estriol reported by Layne et al. (1958) was in all likelihood mainly 16-epiestriol.

The first isolation of estrogens from avian material in a crystalline form was done by MacRae, Zaharia and Common (1959). The characterization was done by means of infrared spectrophotometry and the estrogen isolated from the droppings of laying hens proved to be estradiol-17β. The estrogens subsequently isolated and identified from avian material are presented in Table V and VI.

In the last five years quantitative work on estrogen excretion has been done. The method first described by Brown (1955) has been applied to hens' urine in order to determine estrone; and modifications incorporating thin-layer chromatography have been applied to the determination of estradiol-17β and the cis-estriol (16-epiestriol + 17-epiestriol) fraction. Data for the estrone content of hen's urine during laying and non-laying periods have been reported by Common, Ainsworth, Hertelendy and Mathur (1965). The urinary excretion of estrone and the cis-estriol fractions has been reported by Mathur, Anastassiadis and Common (1966). Mathur and Common (1966) have observed diurnal variation in the 6-hour urinary estrone levels in the laying hen and have suggested a correlation of this variation with ovulation. More recently Mathur and Common (1968a) have determined the levels of estradiol-17β in the laying and non-laying bird and have observed that estradiol-17β is quantitatively the major estrogen in the non-laying bird, whereas in the laying bird the amounts of
<table>
<thead>
<tr>
<th>Estrogen</th>
<th>Source</th>
<th>Criteria</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol-17β</td>
<td>Mixed excreta</td>
<td>Paper chromatography, M.P., I.R. spectra</td>
<td>MacRae, Zaharia &amp; Common (1959)</td>
</tr>
<tr>
<td>16-epiestriol</td>
<td>Urine</td>
<td>TLC of phenol and 5 derivatives in different systems, M.P. of phenol and 4 derivatives</td>
<td>Hertelendy &amp; Common (1964)</td>
</tr>
<tr>
<td>Estradiol-17β</td>
<td>Urine</td>
<td>TLC of 3-methyl ether and 2 derivatives in 3 different systems, M.P. of 3-methyl ether and acetate of 3-methyl ether</td>
<td>Hertelendy, Taylor, Mathur &amp; Common (1965)</td>
</tr>
</tbody>
</table>
TABLE VI

Other isolations of estrogens from avian material

<table>
<thead>
<tr>
<th>Estrogen</th>
<th>Source</th>
<th>Criteria</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrone</td>
<td>Mixed excreta</td>
<td>Paper chromatography, colour reactions.</td>
<td>Hurst, Kuksis &amp; Bendell (1957)</td>
</tr>
<tr>
<td>Estradiol-17β</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estriol*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estrone</td>
<td>Ovarian extracts</td>
<td>Paper chromatography, Kober reaction U.V. spectra</td>
<td>Layne, Common, Maw &amp; Fraps (1958)</td>
</tr>
<tr>
<td>Estradiol-17β</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estriol*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estriol</td>
<td>Urine</td>
<td>TLC of phenol and 4 derivatives in 3 solvent systems.</td>
<td>Mathur &amp; Common (1967)</td>
</tr>
<tr>
<td>16,17-epiestriol</td>
<td>Urine</td>
<td>TLC of phenol and 3 derivatives in 3 solvent systems.</td>
<td>Mathur &amp; Common (1967)</td>
</tr>
<tr>
<td>Estrone</td>
<td>Blood</td>
<td>Paper chromatography</td>
<td>Layne, Common, Maw &amp; Fraps (1958)</td>
</tr>
<tr>
<td>Estrone</td>
<td>Blood plasma</td>
<td>Double isotope derivative crystallization to constant 3H/14C ratio</td>
<td>O'Grady &amp; Heald (1965)</td>
</tr>
<tr>
<td>Estradiol-17β</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* This identification may be considered uncertain in the light of later work.
estrone and estradiol-17β tend to be nearly equal. O'Grady (1968) has reported the determination of estrone and estradiol-17β in avian blood plasma by a method involving the use of double isotope label derivatives.

3. IDENTIFICATION OF ESTROGENS AS IN VIVO METABOLITES

Some information has been accumulated concerning steroid estrogen metabolism in the fowls. The experimental work has included a) administration of estrogens labelled with $^3$H or $^{14}$C and the examination of urinary conversion products and b) in vitro studies of the metabolism of both labelled and unlabelled steroid estrogens by tissue slices or by cell homogenate fractions. Experiments of the former type are described in this section of the historical review.

MacRae, Layne and Common (1959) administered estradiol-17β-$^{14}$C intravenously to a laying hen and identified estradiol-17β, estrone and estriol together with a fourth conversion product, in the gut and mixed excreta. Later this fourth metabolite was identified as 16-epiestriol by MacRae and Common (1960). The ratio of estriol to 16-epiestriol in the droppings of the laying hen was considerably lower than that reported for human urine (Marrian and Bauld, 1955; Hobkirk et al., 1966).

In an attempt to determine if there were qualitative similarities between the metabolic pathways of estrogens in the laying bird and those in the pregnant women, MacRae, Dale and Common (1960) injected estriol-16-$^{14}$C intravenously into a laying hen. If the metabolic pathways were similar, then labelled estriol would be expected to give rise to 16-ketoestradiol-17β and 16-epiestriol (as is the case in humans; Levitz et al., 1958). The 2- and 16-hydroxyestrones if
present would be destroyed when the urine was subjected to acid hydrolysis prior to extraction. In keeping with this expectation the only radioactive conversion products identified in the urine were 16-ketoestradiol-17β and 16-epiestriol. Ainsworth and Common (1963) subsequently injected 16-ketoestradiol-17β-16-14 C into a non-laying hen. The only radioactive conversion product identified and present in appreciable amounts was 16-epiestriol and radioactive estriol could not be detected. The identifications were based on paper chromatography and crystallization of the phenols and their acetates to constant specific activity. This clearly demonstrated that although there are qualitative similarities between the metabolic pathways of estrogens of the fowl and those of women the equilibrium conditions are such that in the former case 16-epiestriol is formed at the expense of estriol, whereas in the latter case estriol is the main conversion product (Levitz, Rosen and Twombly, 1960).

In a further study of the in vivo conversion products of injected estrogens, Ainsworth, Carter and Common (1962) administered estrone-16-14 C intravenously into a laying hen. Ten distinct radioactive zones were observed which were numbered from 1 to 10 in the order of decreasing polarity. Apart from zones 5 and 9 all the others were identified on the basis of their chromatographic mobilities as follows: estriol, 16-epiestriol, 17-epiestriol, 16-ketoestradiol-17β, estradiol-17β, 16-ketoestrone and estrone. 16-Ketoestradiol-17β was further identified by sodium borohydride reduction, which gave mainly 16-epiestriol and a minor spot which may have been due to 17-epiestriol (Ainsworth, 1961).

Zone 8 was identified as 16-ketoestrone on the basis of its chromatographic mobility, the fact that it was completely destroyed along with
Zone 9 when subjected to acid hydrolysis and the finding that borohydride reduction yielded three radioactive reduction products, one of which was thought to be 16-epiestriol and another to be estriol. Attempts were made to identify Zone 7 and 8 with estradiol-17α, however, these proved to be negative. Zone 7 was identified as estradiol-17β. There was no evidence for the presence of 2-methoxyestrone, equilin or equilenin. [As will be shown in the present thesis, there are strong grounds for regarding as erroneous the foregoing conclusions about the identity of the materials of Zone 8 and as to estradiol-17α not being among the conversion products of injected estrone.]

Ainsworth, Carter and Common (1964) injected 16-epiestriol-16-14C and identified 16-epiestriol and 16-ketoestradiol-17β as the major radioactive conversion products. Two minor radioactive products were not identified but neither of these was estriol, 17-epiestriol or 16β-hydroxyestrone. Hertelendy and Common (1965) have reported the identification of estriol, 16-epiestriol, 17-epiestriol, 16,17-epiestriol as urinary conversion products of estradiol-17β-16-14C injected intravenously into a non-laying hen.

There have been a few reports on the uptake of labelled estrogens by the domestic fowl. Even though the metabolites of estrogens in the various tissues have not been identified rigorously, these papers will be reviewed briefly in this section.

Johnsson and Terenius (1965) studied the uptake of estradiol-17β-6,7-3H and labelled hexestrol. One or other of the above mentioned compounds was administered to week-old chicks and the animals killed at various time intervals. The amounts of radioactivity in the oviducts
and other organs were measured. The uptake of radioactivity by the oviducts was twice as much as that by the skeletal muscles and there was no evidence of saturation. A distinct parallelism exists between mammalian uterus and hen oviducts as regard such uptakes.

Serchi, Mascia, Capone, Angius and Pacciani (1966) studied the metabolism of estrone-4-14C in the chicken. The ether- and water-soluble radioactivities in the liver, digestive tract, heart, ovaries and in the residual animal and the urine and feces were studied 5 days following injection. The activity lost in the first 24 hours was 28.9 percent. On successive days radioactivity lost reached 80% and was maximal at 87.6% by 120 hours after injection. In acid-hydrolysed tissue extracts the ratio ether-soluble to water-soluble radioactivity was 2:1, and this ratio decreased to 1:30 on the 4th and 5th day.

Martella (1967) has reported on the uptake of estrone-4-14C by 15-day old chicks. Animals were killed 24 hours, 48 hours and 96 hours after injection and the radioactivities in various organs were determined. In urine and feces (presumably the mixed excrement), the radioactivity increased steadily up to 96 hours. In the liver homogenate the maximal level was reached at 48 hours and there was a slow decrease up to 96 hours, after which the radioactivity decreased rapidly. The radioactivity of the ovary homogenates displayed a steady increase with a maximum at 120 hours. The author has advanced the hypothesis that the hormone is conjugated with a protein, the conjugate being water-soluble.

A more definitive study has been made by Hawkins and Taylor (1967). They studied the metabolites of estradiol-17β-6,7-3H four minutes after intravenous injection. Of the total radioactivity taken up, the greatest
proportion accountable as free steroid occurred in the follicular wall. The blood plasma contained approximately equal amounts of free and conjugated steroids. The free estrogen fraction of all tissues studied contained more than one compound, the composition ranging from 86% estradiol-17β in the liver to virtually no estradiol-17β in the follicular wall. An unidentified compound constituted a large portion of the free steroid in the follicular wall. It was demonstrated that this unidentified compound was neither 16,17-epiestriol nor estriol. Hawkins and Taylor have suggested that the unknown compound may be 16-epiestriol.

Mulay, Carter and Common (1968) attacked the problem of the nature of conjugation of estrogens by injecting estrone-4-14C or estradiol-17β-4-14C intramuscularly into three hens and subjecting the urine to a series of partitions with solvents before and after enzymatic hydrolysis. They were able to show that approximately 85% of the urinary radioactivity occurred in conjugated forms. The proportion present as presumptive glucosiduronates did not exceed 2 to 3 percent. Furthermore, their results provided evidence that a high proportion of the urinary steroid estrogens was present as double conjugates. Mathur and Collins (1968) have reported that the hens' urinary estrogens are largely in the form of single and double esters of sulfuric acid. Their evidence was based on chromatographic studies and various enzymic hydrolyses.

4. IDENTIFICATION OF ESTROGENS AS IN VITRO METABOLITES

In vitro experiments based on the use of tissue slices homogenate fractions and enzyme preparations have yielded an appreciable body of
information relating to the metabolism of steroid estrogens by the fowl. Mitchell and Hobkirk (1959) demonstrated that an appreciable amount of radioactive estradiol-17\(\beta\) was converted to labelled estriol in vitro by liver slices from laying hens. These workers used counter-current techniques for separation of steroids. Their experiments constitute the first published report on the in vitro metabolism of steroid estrogens by avian tissue.

Ozon and Breuer (1965) incubated the following estrogens with liver slices from 10-20 day old chicks and immature pullets: estrone, estradiol-17\(\alpha\), estradiol-17\(\beta\), 16-ketoestradiol-17\(\beta\) and 16\(\alpha\)-hydroxyestrone. They used paper chromatography, thin-layer chromatography, preparation of 3-methyl ethers, the Folin-Ciocalteu reaction, the Kober reaction and microsublimation for separation and identification of the conversion products. These results are presented in Table VII. They have demonstrated the presence of the following enzymes in the chicken liver: 16\(\alpha\)-, 16\(\beta\)-, 17\(\alpha\)-, and 17\(\beta\)-hydroxysteroid oxidoreductases as well as 16\(\alpha\)- and 16\(\beta\)-hydroxylases. Ozon and Breuer (1965) have also located many of these enzymes in the microsomal fraction from the liver. The hen liver has been shown, therefore, to possess the same enzymes as the human liver. However, 16-epiestriol is quantitatively more significant than estriol in the hen; and it is of interest to note in this connection that Ozon and Breuer (1965) have found that the 16\(\beta\)- and 17\(\beta\)-hydroxysteroid oxidoreductases as well as 16\(\beta\)-hydroxylase enzymes were the most active enzymes in the chicken liver. Ozon (1965) also demonstrated that the liver of chicken embryo has very active 16\(\alpha\)- and 16\(\beta\)-hydroxylases. More recently, Renwick and Engel (1967) have reported
### TABLE VII

Conversion of phenolic steroids by chicken liver *in vitro*

(Entire table cited from Ozon & Breuer, 1965)

<table>
<thead>
<tr>
<th>Steroid incubated</th>
<th>Steroid identified</th>
<th>Amounts µg.</th>
<th>Enzymes present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrone</td>
<td>Estradiol-17α*</td>
<td>10</td>
<td>17α-ol-OR**</td>
</tr>
<tr>
<td></td>
<td>Estradiol-17β</td>
<td>30</td>
<td>17β-ol-OR</td>
</tr>
<tr>
<td>Estradiol-17α</td>
<td>Estrone*</td>
<td>15</td>
<td>17α-ol-OR</td>
</tr>
<tr>
<td></td>
<td>Estradiol-17β</td>
<td>10</td>
<td>17α/17β-ol-OR</td>
</tr>
<tr>
<td>Estradiol-17β</td>
<td>Estrone*</td>
<td>40</td>
<td>17β-ol-OR</td>
</tr>
<tr>
<td></td>
<td>Estradiol-17α*</td>
<td>10</td>
<td>17β/17α-ol-OR</td>
</tr>
<tr>
<td></td>
<td>16-Epiestriol</td>
<td>5</td>
<td>16β-Hydroxylase</td>
</tr>
<tr>
<td></td>
<td>Estriol</td>
<td>2</td>
<td>16α-Hydroxylase</td>
</tr>
<tr>
<td>16-Ketoestradiol-17β</td>
<td>16-Epiestriol</td>
<td>30</td>
<td>16β-ol-OR</td>
</tr>
<tr>
<td></td>
<td>Estriol</td>
<td>20</td>
<td>16α-ol-OR</td>
</tr>
<tr>
<td>16α-Hydroxyestrone</td>
<td>17-Epiestriol*</td>
<td>25</td>
<td>17α-ol-OR</td>
</tr>
<tr>
<td></td>
<td>Estriol*</td>
<td>30</td>
<td>17β-ol-OR</td>
</tr>
</tbody>
</table>

* Isolated by microsublimation

** OR = oxidoreductase
the partial purification of 17α- and 17β hydroxysteroid oxidoreductases from chicken liver.

Raud and Hobkirk (1967) incubated 16-epiestriol-16-14C and estriol-6,7-3H separately with homogenates of liver from laying hens. Identification of conversion products was done by paper chromatography and crystallization to constant specific activity both with and without derivative formation. These workers found that the conversion of estriol to 16-epiestriol was much greater than the conversion of 16-epiestriol to estriol. The major intermediate of these interconversions was shown to be 16-ketoestradiol-17β.

Weniger, Ehrhardt and Fritig (1967) cultured the left ovaries of 7 day female chicken embryos in a medium containing sodium acetate-1-14C. They identified small amounts of estrone and estradiol-17β. Akram and Weniger (1967a) were able to demonstrate the formation of estrone and estradiol-17β in the feminized testicle culture of chicken embryo which had been treated with estrogen. Akram and Weniger (1967b) were also able to demonstrate the formation of estrone and estradiol-17β by the left ovaries of 12-day duck embryos cultured in a medium containing sodium acetate-1-14C. Recently, Weniger (1968) has demonstrated that ovaries from 14-16 day chicken embryos produced estriol in vitro when cultured in a medium containing sodium acetate-1-14C.

Haffen and Cedard (1968) have studied the in vitro metabolism of labelled dehydroepiandrosterone and testosterone by the gonads of chicken embryos. They found that female gonads when cultured in vitro with dehydroepiandrosterone-4-14C formed increasing amounts of estrogens as the gonads were taken from progressively older embryos in the range of 7-10 days'
incubation. Estradiol-17β was the major estrogen produced in all
the stages of embryos used in this study. Male gonads from embryos
in the same age range did not produce significant amounts of estrogens
but the production of labelled testosterone increased progressively with
the appearance of Δ4,5-isomerase and 3β-ol-oxidoreductase. Testosterone
and estradiol-17β were obtained in a state of radiochemical purity.
Female and feminized male gonads were similarly able to aromatize
testosterone-6,7-3H to produce tritiated estrone and estradiol-17β but
the yield was somewhat less than that obtained from dehydroepiandrosterone-
4-14C.

The studies described in the above two paragraphs demonstrate the
presence of enzymes involved in the biosynthesis of steroid estrogens in
chick embryos.

Raud and Hobkirk (1968) have investigated the in vitro biosynthesis
of steroid sulfates by cell-free preparations from tissues of laying hens.
They have located esterifying enzymes in the 105000 x g supernatants of
liver, oviduct and vaginal preparations but the liver preparation was
several times more active than the other tissues. They also found that
the enzymes were more efficient in sulfurylating estrogens than other
steroids such as dehydroepiandrosterone. In other studies Raud and
Hobkirk (1968b) incubated estrone-4-14C and estrone-6,7-3H-3-sulfate
simultaneously with chicken liver homogenates and were able to demonstrate
that the liver homogenates from the fowl were capable of catalyzing a
rapid reduction of estrone to estradiol-17β and of estrone-3-sulfate to
estradiol-17β-3-sulfate, the latter apparently without the removal of the
sulfate group. Raud (1968) has presented evidence to show that the
conversion of estrone to estradiol-17β was more rapid than the conversion of estradiol-17β to estrone. There was no evidence for the formation of 2-hydroxyestrone or 2-methoxyestrone from estradiol-17β and estrone. Attempts to stimulate 2-hydroxylation by treating hens with thyroid activating drugs did not yield any C-2 substituted compounds, thus indicating a lack of C-2 hydroxylating enzymes. Liver tissue homogenates from normal hens did not convert estrone or estradiol-17β to estriol, a noteworthy finding in view of the earlier demonstration by Mitchell and Hobkirk (1959) that hen liver slices can convert estradiol-17β to estriol, but the formation of 16-epiestriol was detected. Liver homogenates from hypothyroid (induced) birds did show the conversion of estrone and estradiol-17β to estriol thereby showing that 16α-hydroxylase was present in the liver of the fowl in such low amounts that under normal conditions it cannot be detected. Incubation of 16-ketoestradiol-17β-16-14C with liver tissue homogenates gave rise to both 16-epiestriol and estriol. There were some indications for the presence of 16-ketoestrone in small amounts, but no positive identification of this compound was attained.

5. METABOLISM OF ESTROGENS IN CERTAIN AVIAN SPECIES

Estrogen metabolism in the avian species has been studied primarily in the domestic fowl. There is a remarkable general similarity between the metabolism of estrogens in the domestic fowl and women. The metabolism of estrogens in the fowl is as complex as the metabolism of estrogens in women. Thus far 16α- and 16β- hydroxyestrones have not been identified either as in vivo or in vitro conversion products in the hen, hence the precise series of reactions leading to the formation of estriol epimers could not be compared with those in humans. Recently, however,
Mathur and Common (1968b) have investigated this problem by injecting a mixture of 17β-estradiol-17α-3H and 17β-estradiol-4-14C into a hen and studied the ratio of 3H to 14C in the conversion products. They showed that the tritium label was retained by the 16-epiestriol and 16-ketoestradiol-17β, thereby, demonstrating that 16-epiestriol was formed by a direct hydroxylation of estradiol-17β in contrast to the situation in humans, where estrone formation has been shown to be an obligatory step in the formation of estriol (Fishman et al., 1960). These results also explain the apparent absence of 16α- and 16β-hydroxyestrone, since the formation of 16-epiestriol does not occur through the estrone intermediate, therefore the 16-hydroxyestrone would not be intermediates in the 16-epiestriol biosynthesis. This does not mean that there may be no 16-hydroxyestrone in the hens' urine since small amounts could readily arise from 16-ketoestradiol-17β which has been shown to be present as in vivo conversion product of estrone. The metabolic pathway in the domestic fowl differs in four important aspects from the metabolic pathway in women. First, 16-epiestriol is the major triol in the avian species as opposed to estriol in women. Second, the biosynthesis of the major triol in the domestic fowl is different from that in women; 16-epiestriol is formed by a direct hydroxylation of estradiol-17β in the former, whereas estriol is formed through the estrone and 16α-hydroxyestrone intermediates in the latter. Third, the hen appears to lack the enzymes necessary to carry out C-2 substitution reactions, whereas C-2 substitution reactions are significant in the metabolism of estrogens in women. Fourth, Ozon and Breuer (1965) have demonstrated the conversion of estrone to estradiol-17α and vice-versa.
in vitro in the hen, whereas estradiol-17α formation has not been demonstrated in normal women (Watson and Marrian, 1958; Schott and Katzman, 1964). Breuer and Schott (1966), however, have demonstrated the conversion of estradiol-17α to estrone both in vivo and in vitro. The presence of 17α-hydroxysteroid oxidoreductase has been well established in humans as has been described elsewhere in the historical review.

Further differences exist between the human and the domestic fowl in so far as the latter a) appears not to excrete any appreciable proportion of steroid estrogen in conjugation with glucuronic acid and b) appears to excrete major proportion of her estrogens in the form of single and double conjugates with sulfuric acid, as discussed above.

The intermediary metabolism of estrogens in the domestic fowl is summarized in Figure IV, based on the in vivo and in vitro evidence presented in the preceding two sections. This scheme is essentially based on the scheme given by Ozon and Breuer (1965) but includes the more recent findings, particularly, the identification of 16,17-epiestriol from the urine of hens.
Figure IV: The intermediary metabolism of estrogens in the domestic fowl.

--- Solid lines represent reactions which have been shown to occur both in \textit{in vivo} as well as \textit{in vitro} experiments.

---- Broken lines represent reactions which have been shown to occur in \textit{in vitro} experiments only.

\( d \) Solid line with 'd' represent reactions which have been shown to occur in \textit{in vivo} experiments only.

\( a \) Estrogens isolated from hen urine.

\( b \) Estrogens isolated from mixed excreta.

\( c \) Estrogens identified as radioactive conversion products in \textit{in vivo} experiments.

\( e \) 16α-Hydroxyestrone has thus far not been identified either as \textit{in vivo} or \textit{in vitro} conversion product.

\( f \) Identification of 16-Ketoestrone doubtful (see Chapter VI)
D. OCCURRENCE OF ESTROGENS IN VERTEBRATES OTHER THAN MAMMALS AND AVES.

Although it has been known for a long time that extracts from fish eggs and ovaries contained materials which produced estrus responses, when bioassayed in mammals, it is only in the last decade that several groups of workers have undertaken the identification of steroid estrogens elaborated by the gonads of the lower vertebrates. The literature concerning the occurrence of estrogens in amphibians, reptiles and fishes is now considerable and no attempt is made in this thesis to cover this part of the subject. References are given to the following recent review papers. Chieffi (1966) has reviewed the occurrence of steroids in the gonads of nonmammalian vertebrates and the sites of their biosynthesis. Another review on the isolation and identification of steroid hormones in the lower vertebrates has been provided by Ozon (1966). Gettfried (1964) has reviewed the literature concerning fish.
CHAPTER II

MATERIALS AND METHODS
A. GENERAL MATERIALS AND METHODS

1. REFERENCE STANDARDS

a. Estrone (Steroid Laboratories, Box 247, Montreal) was re-crystallized from aqueous ethanol. The recrystallized material was chromatographically homogenous in three systems and had m.m.p. 260°C.

b. 16-Ketoestrone was presented by Dr. D.S. Layne, University of Ottawa, Ottawa. The reference material was used without any further purification.

c. Estradiol-17α (Steraloids, Pawling, N.J., U.S.A.) was re-crystallized from aqueous ethanol. The purified material was chromatographically homogenous in three systems. It has two distinct micromelting points, the first in the range 208-212°C and the second in the range 221-223°C. Kühnert-Brandstatter, Junger and Kofler (1965) have described the polymorphism of estradiol-17α.

d. Estradiol-17β (Sigma Chemical Co., St. Louis, Mo. U.S.A.) was purified by TLC on silica gel G in system C (see below). The purified material was chromatographically homogenous in three systems and had m.m.p. 176-179°C.

e. 16-Epiestriol (Steraloids Inc., Pawling, N.J., U.S.A.) was purified by recrystallization from aqueous ethanol. The purified material was chromatographically homogenous (TLC) and had m.m.p. 275-277°C.

f. 17-Epiestriol was donated by Dr. J. Fishman, Montifiore Hospital, New York, N.Y., U.S.A. It was used as received.

g. Estriol (Steraloids Inc., Pawling, N.J., U.S.A.) was purified by fractional microsublimation. The purified material was chromatographically
homogenous and had m.m.p. 280°C

h. 16-Ketoestradiol-17β (Steraloids Inc., Pawling, N.J., U.S.A.) was used as received.

i. Estrone acetate (Steraloids Inc., Pawling, N.J., U.S.A.) was used as received, although there was a minor secondary spot when chromatographed.

j. Estriol triacetate (Steraloids Inc., Pawling, N.J., U.S.A.) was chromatographically homogenous and was used without any purification.

k. Estradiol-17β diacetate (Steraloids Inc., Pawling, N.J., U.S.A.) was chromatographically homogenous and was used as such.

l. Estradiol-17β-3-methyl ether (Sigma Chemical Co., St. Louis, Mo., U.S.A.) was chromatographically homogenous and was used as received.

m. The following derivatives were prepared as described in part 8 of the methods section in this chapter. The acetate of estradiol-17β-3-methyl ether and of estradiol-17α-3-methyl ether was prepared by acetyllating the 3-methyl ethers of the diols. Estradiol-17α-3-methyl ether was prepared by methyating reference estradiol-17α according to Brown's procedure. Two methylation products were observed when the methylation product was chromatographed. The major product was due to the 3-methyl ether and the minor spot was believed to be due to the dimethyl ether. The diacetate of estradiol-17α was prepared by acetylvation of the reference estradiol-17α. Only one product was observed on chromatography of the acetylation product.

2. SOLVENTS

Benzene (thiophene free), chloroform, A.R. grade, n-hexane A.R. grade,
methanol, spectranalyzed grade and dimethyl sulfoxide, spectranalyzed grade were obtained from Fisher Scientific Co., Montreal, P.Q.

Ethyl acetate and cyclohexane, laboratory grade, were obtained from British Drug Houses Ltd., Toronto, Ont.

All above solvents except methanol and dimethyl sulfoxide were distilled just before use.

Diethyl ether U.S.P. was obtained from Fisher Scientific Co., Montreal, P.Q. It was freed from peroxides by shaking with 0.3 M FeSO₄ solution in 0.4 N H₂SO₄, washing several times with water and then distilling in the boiling range 33-35°C.

Absolute ethanol U.S.P. was obtained from Consolidated Alcohols Ltd., Montreal, P.Q. Last traces of moisture were removed by refluxing with 5 g NaOH and 5 g Zn metal for each 100 ml for 12 hours and then distilling twice to within narrow limits, B.P. 78.2°C. Last traces of moisture were also removed by a method described in a paper by Givner, Bauld and Vagi (1960) for alcohol used in the Girard separations.

3. CHEMICALS

All chemicals used were reagent grade unless stated otherwise.

Girard P reagent was obtained from Fisher Scientific Co., Montreal, P.Q. The purification was done as described in a paper by Givner et al. (1960).

Medical x-ray film, Kodak liquid x-ray developer and fixer were obtained from Picker x-ray engineering Ltd., Montreal, P.Q.

4. RADIOCHEMICALS AND MATERIALS FOR COUNTING

Estradiol-17β-4-¹⁴C (31.8 mc/mM), 10 μC in 2% methanol solution and
estrone-4-^{14}C (33.7 mc/mM), 10 μC in 5% methanol solution, were obtained from Radiochemical Centre, Amersham, England. The radioactive material was purified by thin-layer chromatography, unless stated otherwise in the experimental section.

Estradiol-17α-6,7-^{3}H was donated by Dr. D.S. Layne, University of Ottawa, Ottawa. It was purified immediately before use by TLC in system X (double development).

^{14}C-Toluene and ^{3}H-toluene for internal standard, PPO (2,5-diphenyloxazole) and POPOP (1,4-bis-2,5-phenyloxazolyl benzene) were obtained from Packard Company, La Grange, Ill., U.S.A. Scintillation grade toluene was obtained from Nuclear Enterprises, Winnipeg, Manitoba.

5. ENZYME PREPARATIONS

The enzyme preparation which is commercially known as 'Ketodase' was purchased from Warner-Chillcott Co., U.S.A. Each ml. of 'Ketodase' contains 5000 Fishman units of β-glucuronidase buffered in acetate to pH 5.

The enzyme preparation was 'Aryl sulfatase' as supplied by Sigma Chemical Co., St. Louis, Mo., U.S.A. The suppliers described it as 'Sigma type II from crude limpet powder, contains 760,000 F.U./g β-glucuronidase and 10 sulfatase units/mg. phenol sulfatase'.

6. COLOUR REAGENTS

Polin and Ciocalteu reagent, Laboratory reagent grade, was obtained from British Drug Houses Ltd., Toronto, Ont.

2% H_{2}SO_{4} in 50% aqueous ethanol was also used to visualize steroid
estrogens, particularly the estrogen derivatives.

7. CHROMATOGRAPHIC EQUIPMENT

Silica gel G according to Stahl (Merck, Darmstadt, West Germany) was obtained from Brinkman Instruments Inc., Long Island, N.Y. and Canlab, Montreal, P.Q.

The thin layer chromatography outfit was purchased from Quickfit Instruments, England.

B. EXPERIMENTAL PROCEDURES

1. COLLECTION AND STORAGE OF URINE SAMPLES.

a. In vivo experiments

Mature Rhode Island Red hens were surgically modified so as to provide exteriorized ureteral openings. The surgical procedure was that of Dixon and Wilkinson (1957) as modified by Ainsworth (1965). The radioactive material dissolved in either 2 ml. propylene glycol or dimethyl sulfoxide was injected intramuscularly into the breast muscle of the hen. The radioactive steroid injected in the various experiments is indicated in the appropriate sections of the thesis. The urine voided subsequent to the injection were collected for a maximal period of 7 days, but normally collections were made for two or three days only. For collection of urine, antibiotics were dusted into the collection tube. In the earlier experiments 125,000 units of penicillin G was dusted into the tube at each collection. In the later experiments 100 mg each of aureomycin and streptomycin was dusted into the tube at each collection. The urine collections were stored in polythene bottles at -15°C pending analysis.
b. Ad hoc experiments

A number of ad hoc experiments were done to eliminate the possibility of conversion occurring after excretion of the urine as a result of microbiological action. In these experiments, radioactive steroid estrogen was purified just before use and was then added to the collection tube directly as a methanolic solution. Different antibiotics were added twice daily at collection time. Only 24-hour collections were made with different antibiotic. See Chapter III for details.

2. FILTRATION

The urine sample was filtered through a Buchner funnel. The solid urates were further extracted by grinding in a mortar with small volumes of water. The volume of the filtered urine plus washings was made up to 500 ml or other convenient volume.

3. ENZYMATIC HYDROLYSIS

a. Hydrolysis with 'Ketodase'

The urine sample was buffered with 0.5 M acetate buffer adjusted to pH 4.5 with glacial acetic acid (4.1 g of sodium acetate anhydrous and 135 mg of potassium phosphate, dibasic, was added to 100 ml of urine sample). The enzyme 'Ketodase' was added at the rate of 500 F.U. per ml of urine, which was then incubated for 24 hours at 37°C. The optimum conditions for enzymatic hydrolysis with β-glucuronidase preparation have been discussed by Beling (1963) and Nakamura and Kushinsky (1963).

b. Hydrolysis with 'Sulfatase'

The urine sample was buffered with 0.15 M acetate buffer adjusted to
pH 5 with glacial acetic acid (1.23 g sodium acetate, anhydrous, was added to 100 ml urine sample). The enzyme 'Arylsulfatase' was added at the rate of 2 sulfatase units per ml of urine, which was then incubated for 24 hours at 37°C. The optimum conditions of enzymatic hydrolysis with phenolsulfatases is discussed by Adlercreutz (1962). The hydrolysis procedure was slightly different in Experiment III (Chapter IV) and will be described later.

4. SOLVOLYSIS

Solvolysis was performed as described by Burstein and Lieberman (1958). Sodium chloride at the rate of 20 g per 100 ml was added to the sample, which was then adjusted to pH 1.0 with concentrated HCl acid and extracted at once with equal volume of ethyl acetate (within 20 minutes). Two ml of glacial acetic acid was then added to the organic phase and it was incubated for 24 hours at 37°C. The organic phase was then washed with 4% NaHCO₃ solution (2 x 1/10 vol.) and then washed with water (2 x 1/10 vol.) and finally dried over anhydrous sodium sulfate. The organic phase was then evaporated in a rotatory evaporator and the residue taken up in spectrana1yzed methanol.

5. THIN-LAYER CHROMATOGRAPHY

a. Preparation of thin-layer plates

Glass plates (20 x 20 cm) were coated with 0.25 mm thick layer of silica gel G. The slurry was prepared by vigorously shaking 30 g of silica gel G with 60 ml of distilled water for two to three minutes. The slurry was then applied to the glass plates with a suitable applicator.
The plates were left on the bench for 20 minutes, then placed in an oven for 45 minutes to 1 hour at 105-110°C. The activated plates were then stored in a desiccator over anhydrous silica gel.

b. **Solvent systems**

The composition of all the solvent systems used in the work described in this thesis are listed in Table VII with appropriate references. All solvents were distilled just before use.

c. **Visualization of steroid estrogens**

The detection of estrogens as phenols was done according to the method of Mitchell and Davies (1954) using the Folin and Ciocalteu reaction (1927). The thin-layer plate was sprayed with a solution made by diluting one volume of the Folin and Ciocalteu phenol reagent with two volumes of distilled water and then exposing the plate to ammonia vapours. A dark blue colour was developed by all the phenolic estrogens.

Estrogen derivatives, such as acetates and methyl ethers, could not be visualized by the Folin-Ciocalteu phenol reagent. These derivatives were detected by spraying plates with \( \text{H}_2\text{SO}_4\)-ethanol\') reagent (2% conc. \( \text{H}_2\text{SO}_4 \) in 50% aqueous ethanol) and heated in a oven at 105°C for 20-25 minutes. Characteristic colours were developed by different estrogen derivatives, ranging from light purple for estriol derivatives to deep pink and orange for the estradiol derivatives.

The separation of the derivatives of reference estradiol-17β, estradiol-17α and some other reference estrogens is shown in Figure V. Note the differences in the colouration of the estrogen derivatives, and the fact that the 3-methyl ether of estradiol-17β is more polar than the 3-methyl ether of estradiol-17α, whereas the diacetate and the acetate of
TABLE VIII
Composition of the solvent systems used for thin-layer chromatography

<table>
<thead>
<tr>
<th>System</th>
<th>Composition by volume</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Cyclohexane:ethyl acetate: ethanol (45:45:10)</td>
<td>Lisboa &amp; Diczfalusy (1962)</td>
</tr>
<tr>
<td>C</td>
<td>Cyclohexane:ethyl acetate (50:50)</td>
<td>&quot;</td>
</tr>
<tr>
<td>0</td>
<td>n-Hexane:ethyl acetate (75:25)</td>
<td>Lisboa (1964)</td>
</tr>
<tr>
<td>X</td>
<td>Chloroform:ethyl ether (60:40)</td>
<td>Sobrevilla, Hagerman &amp; Villee (1964)</td>
</tr>
<tr>
<td>Y₁</td>
<td>Benzene:methanol (95:5)</td>
<td>Ladany &amp; Finkelstein (1963)</td>
</tr>
<tr>
<td>Y₂</td>
<td>Benzene:methanol (99:1)</td>
<td>modification of Y₁ system</td>
</tr>
<tr>
<td>Z</td>
<td>Chloroform (100)</td>
<td>used by the author</td>
</tr>
</tbody>
</table>
Figure V: Photograph illustrating the relative mobilities of reference methyl ethers and acetates of the two estradiol epimers and the acetates of estrone, estriol and 16-ketoestrone in system 2, and the differences in the colour developed by these derivatives when sprayed with \( \text{H}_2\text{SO}_4-\text{ethanol} \) reagent.

1 = Estrone acetate
2 = Estriol triacetate
3 = Acetylation product of 16-ketoestrone
4 = Estradiol-17\( \beta \) diacetate
5 = Estradiol-17\( \alpha \) diacetate
6 = Estradiol-17\( \beta \)-3-methyl ether
7 = Estradiol-17\( \alpha \)-3-methyl ether
8 = Acetate of estradiol-17\( \beta \)-3-methyl ether
9 = Acetate of estradiol-17\( \alpha \)-3-methyl ether

Note: The 3-methyl ether of estradiol-17\( \beta \) is more polar than the 3-methyl ether of estradiol-17\( \alpha \), whereas the diacetate and the acetate of 3-methyl ether of estradiol-17\( \beta \) is less polar than the corresponding derivative of estradiol-17\( \alpha \).
Figure V: Photograph illustrating the relative mobilities of reference methyl ethers and acetates of the two estradiol epimers and the acetates of estrone, estriol and 16-ketoestrone in system 2, and the differences in the colour developed by these derivatives when sprayed with \( \text{H}_2\text{SO}_4-\text{ethanol} \) reagent.

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7 = Estradiol-17\( \alpha \)-3-methyl ether
8 = Acetate of estradiol-17\( \beta \)-3-methyl ether
9 = Acetate of estradiol-17\( \alpha \)-3-methyl ether

Note: The 3-methyl ether of estradiol-17\( \beta \) is more polar than the 3-methyl ether of estradiol-17\( \alpha \), whereas the diacetate and the acetate of 3-methyl ether of estradiol-17\( \beta \) is less polar than the corresponding derivative of estradiol-17\( \alpha \).
3-methyl ether of estradiol-17β is less polar than the corresponding derivatives of estradiol-17α.

d. **Application of sample on thin-layer plates**

The sample being subjected to separation and/or purification was taken up in a minimum volume of methanol and small volumes (generally 10 μl) were withdrawn into a Hamilton syringe (100 μl) attached to a sample applicator and applied to a plate along a line. The tube was washed a few times with small volumes of methanol and the washings were also applied to the plate.

In order to establish the identity of a metabolite, the radioactive compound was applied as small spots by means of a micro-pipette either by mixing the radioactive material with appropriate carrier or by applying reference compounds alongside on lateral strips.

6. **MEASUREMENT OF RADIOACTIVITY**

a. **Measurement of radioactivity in aqueous fractions**

Aqueous fractions, such as filtered urine or aqueous residue after enzymatic hydrolysis, were assayed for radioactivity in the following manner. A sample of 0.5 ml volume was taken, 5 ml absolute ethanol and 10 ml scintillation fluid were added and the counting was performed in triplicate in a Packard Tricarb liquid-scintillation spectrometer model 3003. The quenching was determined by means of an automatic external standard for which an efficiency curve had been plotted. The scintillation fluid was made up as follows:— 3 g PPO and 0.1 g POPOP were dissolved in 1 litre of toluene. The total radioactivity after correcting for quenching was expressed in disintegrations per minute.
b. Measurement of radioactivity in methanolic extracts

A suitable aliquot of the sample was taken and 15 ml of the scintillation fluid was added and counting was done as described above.

In some experiments the quenching factor was determined by using an internal standard. In such instances 10 µl of either $^{14}$C-toluene or $^3$H-toluene was added to each individual vial and these were counted as before.

c. Assay of radioactivity on thin-layer plates

Measurements of radioactivity in the different zones separated by thin-layer chromatography were done in the following manner so as to eliminate elution from the silica gel. Different zones of interest were scraped into separate vials. The silica gel was suspended in a thixotropic gel according to a modification of a method described by Snyder and Stevens (1962). The modification consisted of filling each vial with Cab-O-sil M-5 silica gel up to the neck of the vial and then adding 15 ml of scintillation fluid (5 g PPO + 0.1 POPOP per litre of toluene). This modification has definite advantages over the original method described by Snyder and Stevens (1962). According to their method, a thixotropic scintillation gel is prepared by dissolving 4 g of Cab-O-sil M-5 silica gel in 100 ml of scintillation fluid (5 g PPO + 0.1 g POPOP per litre toluene) and then adding 15 ml of this gel to each vial. Such a gel is difficult to pour and it is difficult, furthermore, to add exactly 15 ml of the gel. By filling the vial with Cab-O-sil M-5 in the dry state up to the neck, approximately the same amount of Cab-O-sil M-5 is added as in the original method (0.6 g per vial). The exact amount of scintillation fluid can then be added without further difficulty.
The vials were capped with Scotch tape and trimmed to fit the circumference of the opening before placing the lids and shaking the vials. The samples were counted in a Packard liquid scintillation counter and quenching factor determined by means of an automatic external standard, as described before.

Measurements of separation effected by TLC of metabolites labelled with tritum, which could not be readily radioautographed, was done by scraping serial strips either 3 mm or 5 mm wide into vials and counting them as described above. A histogram was then plotted and the radioactive peaks were compared with the reference standards on pilot strips. The Rf value of the radioactive metabolites were also determined from the histograms.

When only the location of relative positions of the radioactive zones was required, serial strips (5 mm wide) were scraped into vials, 0.2 ml methanol and 15 ml scintillation fluid (3 g PPO + 0.1 g POPOP per litre toluene) was added to each vial and counting was done as described for methanolic extracts. This method of counting afforded a rapid method for locating radioactive zones in the thin-layer plates but it cannot be used for determining the exact proportions of radioactivity in each zone.

7. RADIOAUTOGRAPHY

Radioautography proved to be an extremely useful tool in locating radioactive zones on thin-layer plates without any loss of radioactive sample. The developed chromatoplate was placed in a cassette and a medical x-ray film was cut to the appropriate size and placed in contact with the plate. The cassette was left in the dark for an appropriate period of time. The time of exposure was determined empirically. The
x-ray film was then developed and fixed in a Kodak developer and fixer. The cassettes used for preparation of radioautograms were constructed according to the description of Richardson, Welicky, Batchelder, Griffith and Engel (1963).

There are certain limitations in the use of radioautography which restrict its use. Firstly, radioautography can be readily done only with radio-isotopes which emit a sufficiently energetic β-particle, such as carbon-14. Radio-isotopes which emit a weak β-particle, such as tritium, require the presence of a large amount of radioactivity. Secondly, when small amounts of radioactivity are present in a particular zone or spot, extremely long time exposures are necessary during which period the radioactive material on the plate may become oxidized and thus of little use for identification purposes.

In experiments involving the use of tritium, radioautography was not attempted. Location of radioactive zones on thin-layer plates was done by the method described in the above section.

8. SEPARATION OF KETONIC AND NON-KETONIC ESTROGENS

The separation of ketonic and non-ketonic metabolites of radioactive estrogens was done by the Girard reaction according to the procedure outlined by Givner, Bauld and Vagi (1960). The ketonic estrogens formed a water-soluble complex with Girard P reagent, therefore, the non-ketonic estrogens could be readily extracted from the aqueous solution with an organic solvent.

9. PREPARATION OF DERIVATIVES

a. Acetylation
One ml of dry pyridine and 1.0 ml of acetic anhydride were added to a glass-stoppered tube containing the estrogen. The tube was stoppered and left overnight at room temperature. The following morning, the excess of pyridine and acetic anhydride was removed by evaporating the mixture to dryness under a slow stream of nitrogen. The residue was taken up in methanol.

b. Methylation

The methylation of estrogen was done, as described by Brown (1955), by addition of dimethyl sulfate in a borate buffer at pH 10. The excess of dimethyl sulfate was oxidized with H₂O₂ (30% by volume). The methyl ethers were extracted into suitable organic phase and the organic phase was washed with water to neutral pH. The methyl ethers were freed from the organic phase and the residue taken up in methanol.

10. IDENTIFICATION OF PRESumptIVE ESTROGEN BY REVERSE ISOTOPE DILUTION

The presumptive radioactive metabolite was mixed with approximately 25-30 mg of the appropriate carrier and crystallized from aqueous ethanol. The specific activity of the crystals and the mother liquor were determined. This was repeated two to three times till the specific activities of the crystals and the mother liquor were identical within the limits of experimental error. Axelrod, Matthijssen, Goldzieher and Pulliam (1965) have discussed the criteria for the identification of micro-quantities of steroids by recrystallization to constant specific activity.

In some experiments a derivative of the phenolic estrogen was prepared and the derivative was crystallized to constant specific activity.
The phenolic radioactive metabolite was mixed with 20-25 mg of appropriate carrier and the mixture was acetylated as described before. The acetate was crystallized from aqueous ethanol and taken to constant specific activity.
CHAPTER III

PRELIMINARY AD HOC EXPERIMENTS
A. EXPERIMENT I

1. OBJECT.

To determine whether radioactive steroids used in in vivo studies are converted to other radioactive compounds during collection and storage.

2. EXPERIMENTAL

Estrone-4-$^{14}$C was purified by thin-layer chromatography prior to use. Purified estrone-4-$^{14}$C (3.58 x 10$^6$ dpm) was added to the collection tube. Urine was collected for a 24-hour period and stored. The following scheme was used for processing the urine.

Filtered urine (550 ml) -- -- -- -- -- count
Extract with 2 x 1 vol. CHCl$_3$ -- -- -- CHCl$_3$ evaporated and residue taken up in methanol -- -- count
Aqueous residue count
Residue subjected to TLC in system C
Radioautography of plate.

3. RESULTS AND DISCUSSION

The amount of radioactivity recovered in the urine was only 48.5% (1.738 x 10$^6$ dpm) of the radioactivity added to the collection tube. This recovery was much lower than expected, however, the possibility of the radioactive material being absorbed by the tube when it is added directly to the collection tube cannot be eliminated.
Since unconjugated radioactive estrone had been used, all the radioactivity would be expected to be removed from the urine by chloroform extractions. In keeping with this expectation, chloroform extractions brought out 92.3% \((1.575 \times 10^6 \text{ dpm})\) of the urinary radioactivity. Only 1.6% \((2.8 \times 10^4 \text{ dpm})\) of urinary radioactivity was recovered in the aqueous residue. A total recovery of 93.9% is considered to be reasonable for such extractions, in which troublesome emulsions are formed.

Thin-layer chromatography of the residue from the chloroform extracts and subsequent radioautography indicated that, apart from the major estrone band, several other radioactive bands were formed which were faint but distinct. Of these bands, two corresponded in their \(R_f\) values with estradiol-17\(\alpha\) and estradiol-17\(\beta\). The proportions of radioactivity in the different bands were counted and it was found that all the radioactive bands other than estrone amounted to only 2.2% of the radioactivity on the thin-layer plate. The bands corresponding to estradiol-17\(\alpha\) and estradiol-17\(\beta\) were scraped from the plate separately and eluted with methanol. The eluates were applied to three thin-layer plates and developed in systems A, X and Y\(_1\) along with reference estrogen phenols on lateral strips. Radioautograms of these plates showed that the \(R_f\) values of the radioactive conversion products of estrone-4-\(^{14}\text{C}\) corresponded to estradiol-17\(\alpha\) and estradiol-17\(\beta\) respectively in all the three systems. The eluates from the two bands were then subjected to acetylation and the acetylation product was chromatographed in system Z with estrone acetate, estradiol-17\(\alpha\) diacetate, estradiol-17\(\beta\) diacetate and estriol triacetate reference standards. No radioactivity was found
in the region corresponding to any of the reference acetates. All the radioactivity was found to occur at the point of application. It was clear, therefore, that although the two radioactive bands had Rf values corresponding to estradiol-17α and estradiol-17β in three different systems, they could not be either of the estradiols since neither of them could be acetylated.

In conclusion, it can be stated that a small proportion (2.2%) of estrone-4-14C was converted to other radioactive metabolites, of which two were shown to be neither estradiol-17α nor estradiol-17β. The exact nature of these products was not determined. Further experiments were necessary to determine if these products were formed due to the action of micro-organisms or represented breakdown products of estrone-4-14C in aqueous medium.

B. EXPERIMENT II

1. OBJECT

To determine whether conversion of estrone-4-14C is due to the action of micro-organisms.

2. EXPERIMENTAL.

The procedure by which urine is collected from hens is such that bacterial contamination of urine is very likely. To determine the extent of such a contamination if it does occur, during collection and storage, a bacterial count of the urine was done in the following manner. Two samples of urine were collected, one being collected in the usual manner for a 24-hour period. During the collection of the second sample,
penicillin G (125,000 units) was dusted into the tube twice during the 24-hour period. Both samples were frozen for about the same period as in vivo experiments. Samples were defrosted, and filtered as described elsewhere and the volumes made up to 500 ml. A bacterial count was done for both the urine samples using agar nutrient solution and carrying out incubations at 30°C. The urine sample collected in the absence of penicillin had a bacterial count of 10,000 per ml and the urine sample collected in the presence of penicillin had a bacterial count of 200 per ml. Although the bacterial count was done with one set of urine only, it is evident that there is some bacterial contamination of urine. However, one cannot conclude from the above evidence that the conversion of radioactive estrone to other radioactive metabolites was due to the action of the urinary bacteria.

A time-sequence study of the conversion of estrone-4-$^{14}$C was undertaken to find out if there was noticeable increase in the conversion of estrone to other products with time. The rationale of such an experiment was that if the conversion of estrone were due to micro-organisms, then these would increase over a period of time and thus the conversion of estrone-4-$^{14}$C to other radioactive metabolites would also increase with time.

A urine sample was collected without dusting any penicillin G into the collection tube. It was stored and filtered as described above and the volume made up to 600 ml. Purified estrone-4-$^{14}$C (2.442 x 10$^6$ dpm) was added to the urine and the urine was kept at room temperature. 100 ml of urine was removed at 0, 1, 3, 6, 12, and 24 hours after addition of estrone to urine and processed as described in Experiment (I).
3. RESULTS AND DISCUSSION

The recovery of radioactivity in the chloroform extracts ranged from 92.3% to 96.8%, which recoveries were of the same order as in Experiment (I). The proportions of radioactivity in bands corresponding to reference estrogen phenols are tabulated as percentages of radioactivity on the plate in table IX.

The proportion of radioactivity present in the estrone band ranged from 94.9% to 98.1%, but no decrease in the estrone band was observed with time, as would be expected if appreciable proportions of the estrone had been converted to other radioactive metabolites. The proportions of radioactivity in other zones were similar for urine samples kept at room temperature for different times. A prominent feature of the radioautograms of the separations of CHCl₃ extracts was the presence of two radioactive bands above estrone. These bands were observed neither in in vivo experiments nor in ad hoc experiments where radioactivity was added to the collection tubes. However, they were observed when estrone-4-¹⁴C was subjected to purification prior to use. It can be theorized, therefore, that these bands represent breakdown products of estrone-4-¹⁴C but do not represent conversion products due to action of micro-organisms. Their absence in ad hoc experiments where radioactivity has been added to the collection tube could possibly have been due to a preferential absorption of these breakdown products by the collection tubes.

No further attempt was made to characterize the various bands. It should, however, be noted that the band corresponding in Rf value to
<table>
<thead>
<tr>
<th>Zones</th>
<th>0 hours</th>
<th>1 hours</th>
<th>3 hours</th>
<th>6 hours</th>
<th>12 hours</th>
<th>24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Line of application</td>
<td>0.6%</td>
<td>0.8%</td>
<td>0.5%</td>
<td>0.5%</td>
<td>0.3%</td>
<td>0.5%</td>
</tr>
<tr>
<td>2 Zone corresponding to estriol</td>
<td>0.2%</td>
<td>0.2%</td>
<td>0.2%</td>
<td>0.2%</td>
<td>0.1%</td>
<td>0.2%</td>
</tr>
<tr>
<td>3 Zone between estriol and 16-epiestriol</td>
<td>0.3%</td>
<td>0.3%</td>
<td>0.3%</td>
<td>0.2%</td>
<td>0.2%</td>
<td>0.2%</td>
</tr>
<tr>
<td>4 Zone corresponding to 16-epiestriol</td>
<td>0.2%</td>
<td>0.2%</td>
<td>0.6%</td>
<td>0.2%</td>
<td>0.2%</td>
<td>0.2%</td>
</tr>
<tr>
<td>5 Zone corresponding to 17-epiestriol</td>
<td>0.7%</td>
<td>0.6%</td>
<td>0.7%</td>
<td>0.7%</td>
<td>0.6%</td>
<td>0.7%</td>
</tr>
<tr>
<td>6 Zone between 17-epiestriol and estradiol-17β</td>
<td>0.7%</td>
<td>0.5%</td>
<td>0.4%</td>
<td>0.4%</td>
<td>0.3%</td>
<td>0.2%</td>
</tr>
<tr>
<td>7 Zone corresponding to estradiol-17β</td>
<td>0.4%</td>
<td>0.2%</td>
<td>0.2%</td>
<td>0.2%</td>
<td>0.1%</td>
<td>0.2%</td>
</tr>
<tr>
<td>8 Zone corresponding to estradiol-17α</td>
<td>0.5%</td>
<td>0.3%</td>
<td>0.2%</td>
<td>0.2%</td>
<td>0.2%</td>
<td>0.2%</td>
</tr>
<tr>
<td>9 Zone corresponding to estrone</td>
<td>94.9%</td>
<td>96.0%</td>
<td>96.2%</td>
<td>97.4%</td>
<td>98.1%</td>
<td>96.7%</td>
</tr>
<tr>
<td>10 Zones above estrone</td>
<td>1.7%</td>
<td>1.0%</td>
<td>0.9%</td>
<td>0.9%</td>
<td>0.8%</td>
<td>0.9%</td>
</tr>
</tbody>
</table>

TABLE IX

Proportion of radioactivity in zones corresponding to reference estrogens
reference 17-epiestriol contained 0.7% of the radioactivity on the plate, therefore any future identification of 17-epiestriol as an \textit{in vivo} conversion product in the hen would require a characterization of this breakdown product so as to eliminate the possibility of the 17-epiestriol being a breakdown product of estrone-$4^{14}$C.

One further experiment was done to show that the conversion of estrone-$4^{14}$C to other radioactive metabolites was not due to microorganisms. A urine sample was collected and filtered as described above. It was boiled for thirty minutes to destroy any micro-organisms or enzymes present in the urine and then $3.58 \times 10^6$ dpm of purified estrone-$4^{14}$C was added. The urine was processed as described in Experiment (I). Ninety-nine percent of the radioactivity was recovered in the chloroform extracts. The proportions of radioactivity were similar to those shown in Table IX. Ninety-seven decimal three percent of the radioactivity was present in the estrone band. Two bands above estrone were also observed and these accounted for 1.0% of the radioactivity on the plate, which was a proportion similar to those observed in the time sequence experiment.

This experiment along with the time-sequence experiment demonstrates that although bacteria were present in the urine, they did not convert estrone-$4^{14}$C to other radioactive compounds. The last experiment further demonstrated that the radioactive bands observed represented a breakdown of estrone-$4^{14}$C in an aqueous medium. Attention should be drawn to the fact that in \textit{in vivo} experiments, little if any, estrogen was excreted in the free steroid form, therefore, these \textit{ad hoc} studies did not give the exact measure of the breakdown of estrogen in \textit{in vivo} experiments.
However, such a breakdown would be less for *in vivo* experiments.

Although it had been demonstrated that conversion of estrone-$4^{-14}$C to other radioactive compounds was not due to the action of microorganisms, it was considered desirable to control the growth of bacteria in the urine by means of antibiotics. It was further shown that penicillin G controls the growth of bacteria when dusted into collection tubes during collection. It was observed, however, that during extraction with chloroform, a certain amount of the antibiotic was also extracted and that this antibiotic later interfered in the chromatographic separation. Further experiments were undertaken to find an effective antibiotic which would not interfere thus in the experimental procedure.

C. EXPERIMENT III

1. OBJECT

To find a suitable antibiotic to control bacteria in the urine such that it does not interfere in the experimental procedure.

2. EXPERIMENTAL

Four readily available antibiotics were tested in these experiments. These were as follows:— penicillin G, aureomycin, streptomycin, and sulfaacetamide. Purified estrone-$4^{-14}$C ($1.357 \times 10^6$ dpm) was added to the collection tube and approximately 100 mg of an antibiotic was dusted into the tube twice a day. Such collections were made with all the antibiotics listed and one more collection was made with a mixture of aureomycin and streptomycin. These samples were processed as described for Experiment I.
3. RESULTS AND DISCUSSION

The recoveries of radioactivity in the urine ranged from 51.4% (for sulfacetamide) to 66.1% (for penicillin G) of the radioactivity added to the collection tubes. These recoveries were similar to the recovery of radioactivity in Experiment I. The amount of radioactivity extracted by chloroform ranged from 90.6% (for sulfacetamide) to 94.6% (for a mixture of aureomycin and streptomycin). These recoveries were slightly lower than corresponding recoveries in chloroform extracts in Experiments I and II.

The proportions of radioactivity in bands corresponding to reference estrogen phenols are given in Table X.

The proportions of radioactivity corresponding to reference estrogens in these experiments were similar to those observed in the time-sequence experiment (see Table IX). The bands above estrone were absent in these samples, however, there appeared to be a corresponding increase in the zone which had an Rf value similar to 17-epiestriol. It is likely, therefore, that the breakdown products of estrone-4-$^{14}$C with Rf values greater than that of estrone underwent further changes and gave rise to a breakdown product with an Rf value similar to that of 17-epiestriol. The recovery in the estrone band ranged from 94.4% (for sulfacetamide) to 95.9% (for aureomycin). These results were similar to those in Experiments I and II.

The above results did not indicate any differences in the action of the antibiotic on the breakdown of estrone-4-$^{14}$C. Sulfacetamide was extracted readily by chloroform and interfered considerably in the
TABLE X

Experiment III: Proportions of radioactivity in zones corresponding to reference estrogens

<table>
<thead>
<tr>
<th></th>
<th>Aureomycin</th>
<th>Streptomycin</th>
<th>Penicillin G</th>
<th>Aureomycin + Streptomycin</th>
<th>Sulacetamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Line of application</td>
<td>0.3%</td>
<td>0.3%</td>
<td>0.5%</td>
<td>0.2%</td>
<td>0.4%</td>
</tr>
<tr>
<td>2 Zone corresponding to estriol</td>
<td>0.1%</td>
<td>0.3%</td>
<td>0.5%</td>
<td>0.2%</td>
<td>0.4%</td>
</tr>
<tr>
<td>3 Zone between estriol and 16-epiestriol</td>
<td>0.4%</td>
<td>0.5%</td>
<td>0.8%</td>
<td>0.5%</td>
<td>1.0%</td>
</tr>
<tr>
<td>4 Zone corresponding to 16-epiestriol</td>
<td>0.4%</td>
<td>0.5%</td>
<td>0.6%</td>
<td>0.5%</td>
<td>0.6%</td>
</tr>
<tr>
<td>5 Zone corresponding to 17-epiestriol</td>
<td>1.6%</td>
<td>2.2%</td>
<td>1.8%</td>
<td>2.0%</td>
<td>2.3%</td>
</tr>
<tr>
<td>6 Zone between 17-epiestriol and estradiol-17β</td>
<td>0.7%</td>
<td>0.4%</td>
<td>0.5%</td>
<td>0.6%</td>
<td>0.7%</td>
</tr>
<tr>
<td>7 Zone corresponding to estradiol-17β</td>
<td>0.3%</td>
<td>0.3%</td>
<td>0.3%</td>
<td>0.4%</td>
<td>0.3%</td>
</tr>
<tr>
<td>8 Zone corresponding to estradiol-17α</td>
<td>0.4%</td>
<td>0.2%</td>
<td>0.5%</td>
<td>0.5%</td>
<td>0.3%</td>
</tr>
<tr>
<td>9 Zone corresponding to estrone</td>
<td>95.9%</td>
<td>95.5%</td>
<td>94.6%</td>
<td>95.2%</td>
<td>94.4%</td>
</tr>
</tbody>
</table>
chromatographic separation of the chloroform extract. Penicillin G also interfered with the chromatographic separation, though to a much lesser extent. Aureomycin and streptomycin did not interfere in the separation, although, aureomycin was extracted by chloroform in small amounts.

The use of a mixture of aureomycin and streptomycin for dusting collection tubes to prevent bacterial growth was considered to be most desirable. Either antibiotic, however, could be used for a satisfactory control of micro-organisms.
CHAPTER IV

IDENTIFICATION OF ESTRADIOL-17α AS AN IN VIVO URINARY CONVERSION

PRODUCT OF ESTRADIOL-17β-4-14C AND ESTRONE-4-14C
A. INTRODUCTION

Ainsworth et al. (1962) injected estrone-16-\(^{14}\)C into laying hens and obtained chromatographic evidence for the presence of estrone, estradiol-17\(\beta\), 16-ketoestradiol-17\(\beta\), 16-ketoestrone, 17-epiestriol, 16-epiestriol and estriol. None of these experiments gave any evidence for the presence of estradiol-17\(\alpha\), 2-methoxyestrone, equilin or equilenin. Several estrogens have since been isolated in crystalline form from hen's urine and characterized more fully and these are as follows: estrone (Ainsworth and Common, 1962), estradiol-17\(\beta\) (Hertelendy et al., 1965), 16-epiestriol (Hertelendy and Common, 1964). More recently, estriol and 16,17-epiestriol (Mathur and Common, 1967) have been isolated from hens' urine and on the basis of the thin-layer chromatographic mobilities of the phenols and their derivatives.

For a long time, it was believed that estradiol-17\(\alpha\) was not an \textit{in vivo} conversion product of injected estrone. During the course of experiments investigating the nature of estrogen conjugates in hen's urine (Mulay et al., 1968), the free phenolic fraction which had been obtained by chloroform extraction of urine from hens that had received injections of estradiol-17\(\beta\)-4-\(^{14}\)C was examined by thin-layer chromatography in system C and subsequent radioautography. The radioautograms displayed three strongly radioactive spots which had the same mobilities as estrone, estradiol-17\(\beta\) and 16-ketoestrone together with several weak spots which are not relevant to the present discussion. There was very little radioactivity at the point of application. The Rf value of the radioactive spots corresponding to estrone and estradiol-17\(\beta\) were checked
in two other systems and these had the same mobilities as reference estrone and estradiol-17β in these systems. Similar attempts to check the Rf values of the presumptive 16-ketoestrone were unsuccessful.

The free phenolic fraction was stored in the refrigerator for 6 months and reexamined by the same techniques as those used in the first examination. The radioautograms displayed strongly radioactive spots corresponding to estrone and estradiol-17β, but the radioactive spot presumed to correspond to 16-ketoestrone was no longer present, while a strong radioactive spot appeared at the point of application. These observations suggested that the highly labile compound corresponding in Rf value to reference 16-ketoestrone had undergone alterations to highly polar products during storage. A weakly radioactive spot was observed in the region corresponding to reference 16-ketoestrone. The material from the residual spot was further examined by TLC in system X, which separates reference estradiol-17α and 16-ketoestrone quite satisfactorily. The material from the residual spot corresponded in Rf value to reference estradiol-17α rather than 16-ketoestrone. It was evident, therefore, that at least part of the material which corresponded in Rf value to 16-ketoestrone in system C during the first examination contained a compound which had the same mobility as estradiol-17α in system X.

Ozon and Breuer (1965) published a paper at about the same time as these observations were made, in which they demonstrated the in vitro conversion of estrone to estradiol-17α by chicken liver slices.

The foregoing observations prompted a fuller investigation of the possibility that estradiol-17α may be formed in vivo in the hen.
B. EXPERIMENTAL AND RESULTS

1. EXPERIMENT I

Purified estradiol-17β-4⁻¹⁴C (2.062 x 10⁷ dpm) in propylene glycol was injected into a laying hen and the urine was collected for a 24-hour period. The urine was processed as described below.

a. Outline of procedure for processing urine

Filtered urine (500 ml)

Urine was buffered with 0.5 M acetate buffer pH 4.5 and hydrolysis done with β-glucuronidase as described in Chapter II.

Extract with 2 x 1 vol. CHCl₃ - - - - - - - - - - - - count

Readjust pH and incubate as before

Extract with 2 x 1 vol. CHCl₃ - - - - - - - - - - - - count

Readjust pH and incubate as before

Extract with 2 x 1 vol. CHCl₃ - - - - - - - - - - - - count

Aqueous Residue

count
Bulk CHCl₃ extracts evaporate CHCl₃ and dry residue in desiccator. Girard reaction done with the residue as described in chapter II and extracted with ethyl ether as described before.

Aqueous Ketonic fraction Non-ketonic fraction

count count

subject residue of non-ketonic fraction to TLC

b. Results

The non-ketonic fraction was chromatographed in system X with appropriate reference estrogens on lateral strips and the chromatoplate was radioautographed (exposure time = three days). The presumptive estradiol-17α and estradiol-17β zones were removed separately and the radioactive materials recovered. Small aliquots of the presumptive estradiol-17α and estradiol-17β were methylated and a portion of the methylated product was further acetylated. Samples of presumptive estradiol-17α, presumptive estradiol-17β and their methyl ethers and the acetate of the methyl ethers were subjected to TLC in three different solvent systems with appropriate reference material. The radioactivity
TABLE XI

Experiment I: Chromatographic mobilities of urinary presumptive estradiol-17α and estradiol-17β and their derivatives and mobilities of corresponding reference materials.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Solvent systems</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Estradiol-17α</td>
<td>0.56</td>
</tr>
<tr>
<td>Estradiol-17β</td>
<td>0.54</td>
</tr>
<tr>
<td>Estradiol-17α methyl ether</td>
<td>0.63</td>
</tr>
<tr>
<td>Estradiol-17β methyl ether</td>
<td>0.61</td>
</tr>
<tr>
<td>Acetate of estradiol-17α methyl ether</td>
<td>0.78</td>
</tr>
<tr>
<td>Acetate of estradiol-17β methyl ether</td>
<td>0.77</td>
</tr>
</tbody>
</table>

* Presumptive
+ Reference
on these plates was located by radioautography and the reference material was visualized by the \( H_2SO_4^{-}\text{-ethanol} \) reagent. The Rf values of the presumptive estradiol-17\( \alpha \) and of presumptive estradiol-17\( \beta \) and their derivatives are summarized in Table XI. The Rf values of the corresponding reference material is also given in Table XI.

These results demonstrated that (a) the non-ketonic fraction of the phenolic extract of the urine contained a conversion product easily separable by TLC from estradiol-17\( \beta \); and (b) that this phenol, its methyl ether and acetate of methyl ether had mobilities, in three different solvent systems, which corresponded with the mobilities of reference estradiol-17\( \alpha \) and its appropriate derivatives.

The preliminary observations were insufficient to identify radioactive presumptive estradiol-17\( \alpha \) but they excluded the possibility that the presumptive estradiol-17\( \alpha \) zone could be due to either estradiol-17\( \beta \) or 16-ketoestrone. The inclusion of Girard reaction ensured an effective separation of estradiol-17\( \alpha \) from 16-ketoestrone. Furthermore, if the reaction were incomplete and some 16-ketoestrone remained in the non-ketonic fraction then the initial chromatography of non-ketonic fraction in system X would separate 16-ketoestrone from estradiol-17\( \alpha \). No radioactive zone corresponding to estrone or 16-ketoestrone was observed on the radioautogram, indicating that the Girard reaction was complete.

2. EXPERIMENT II

Estrone-4-\( ^{14}C \) (20.948 \( \times \) 10\( ^{6} \) dpm) was injected into a non-laying hen. The purity of estrone-4-\( ^{14}C \) was checked by TLC and subsequent radioautography and it was found to be at least 99\% pure. Penicillin G
TABLE XII

Experiment II: Recoveries of radioactivity in the urine and certain urinary fractions following the injection of estrone-4-14C into a non-lying hen.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Radioactivity as dpm x 10^-6</th>
<th>percentage of urinary radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total injected</td>
<td>20.948</td>
<td>--</td>
</tr>
<tr>
<td>Urine*</td>
<td>5.989</td>
<td>100.0</td>
</tr>
<tr>
<td>CHCl3 extracts of enzyme hydrolyzed urine</td>
<td>2.485</td>
<td>41.5</td>
</tr>
<tr>
<td>Residual aqueous phase</td>
<td>2.768</td>
<td>46.2</td>
</tr>
<tr>
<td>Pooled CHCl3 extract used for Girard separation</td>
<td>2.446</td>
<td>40.8</td>
</tr>
<tr>
<td>Ketonic from Girard separation</td>
<td>1.928</td>
<td>32.2</td>
</tr>
<tr>
<td>Non-ketonic from Girard separation</td>
<td>0.358</td>
<td>6.0</td>
</tr>
<tr>
<td>Presumptive estradiol-17α</td>
<td>0.021</td>
<td>0.35</td>
</tr>
<tr>
<td>Presumptive estradiol-17β</td>
<td>0.184</td>
<td>3.1</td>
</tr>
</tbody>
</table>

* The recovery of radioactivity in the urine as a percentage of the injected material for the first 24-hour period is 28.6%.

Note: The ratio of estradiol-17β : estradiol-17α for the non-lying hen is 8.7 : 1
(125,000 units) was dusted into the collection tube and the urine was collected and stored as described in chapter II. The urine was processed as described in part (a) of Experiment I, chapter IV. The recoveries of the injected radioactivity and its recovery in various fractions are reported in Table XII. The thin-layer plate on which the non-ketonic fraction of the phenolic extract had been chromatographed (system X) was subjected to radioautography. The radioautogram was used as a guide to scrape the presumptive estradiol-17α zone. This had an advantage over the usual method of using the reference standards on lateral strips as a guide, in so far as any bowing in the separation could be observed readily on the radioautogram and the scraping of the appropriate zone done accordingly. Losses and contamination of the estradiol-17α fraction were minimized by this procedure.

The presumptive radioactive estradiol-17α was mixed with reference estradiol-17α and recrystallized as described in chapter II. Constant specific activity was attained with the third crystallization. The results are summarized in Table XIII.

The final crystals obtained from the crystallization to constant specific activity were used for the preparation of the diacetate, the methyl ether and the acetate of the methyl ether. Small amounts of the phenol and the three derivatives, each corresponding to approximately 20-25 µg of material, were spotted on chromatoplates and chromatographed in suitable solvent systems, then radioautographed and stained with a suitable colour reagent. Control spots of estradiol-17α were also applied on the plates to eliminate the possibility of error arising from chemical blackening of the film. It is well known that large amounts
TABLE XIII

Experiment II: Crystallization of urinary presumptive estradiol-17α to constant specific activity with carrier.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>dpm</th>
<th>mg</th>
<th>specific activity, dpm/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original pool</td>
<td>21,000</td>
<td>25.40</td>
<td>860</td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; crystallization</td>
<td>8,475</td>
<td>17.39</td>
<td>487</td>
</tr>
<tr>
<td>Mother liquor</td>
<td>3,075</td>
<td>4.17</td>
<td>725</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; crystallization</td>
<td>5,864</td>
<td>12.95</td>
<td>453</td>
</tr>
<tr>
<td>Mother liquor</td>
<td>1,221</td>
<td>2.31</td>
<td>529</td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt; crystallization</td>
<td>4,289</td>
<td>9.90</td>
<td>433</td>
</tr>
<tr>
<td>Mother liquor</td>
<td>967</td>
<td>2.18</td>
<td>444</td>
</tr>
</tbody>
</table>
TABLE XIV

Experiment II: Chromatographic mobilities of urinary presumptive estradiol-17α and its derivatives. In each case the mobility of the radioactive material coincided with the mobility of the carrier reference material. Numerical values as Rf.

<table>
<thead>
<tr>
<th>Solvent systems</th>
<th>Derivative</th>
<th>A</th>
<th>C</th>
<th>X</th>
<th>Y&lt;sub&gt;2&lt;/sub&gt;</th>
<th>Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol</td>
<td></td>
<td>0.59</td>
<td>0.44</td>
<td>0.46</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Diacetate</td>
<td></td>
<td>--</td>
<td>0.70</td>
<td>--</td>
<td>0.51</td>
<td>0.61</td>
</tr>
<tr>
<td>3-methyl ether</td>
<td></td>
<td>0.79</td>
<td>0.60</td>
<td>0.73</td>
<td>--</td>
<td>0.34</td>
</tr>
<tr>
<td>Acetate of 3-methyl ether</td>
<td></td>
<td>--</td>
<td>0.78</td>
<td>--</td>
<td>0.58</td>
<td>0.70</td>
</tr>
</tbody>
</table>
of estradiol-17α in contact with x-ray film will cause blackening of the film when exposure is prolonged. No such blackening was observed with the control spots of reference estradiol-17α. The absolute amount of radioactive urinary estradiol-17α in each spot was too small to yield a positive reaction with the colour reagents, hence a complete correspondence in shape and size of the blackening on the radioautograms and the colouration on the corresponding plate provided evidence for the identity of the radioactive material with the reference carrier. The mobilities of the presumptive radioactive estradiol-17α and its derivatives corresponded with the mobilities of the reference carrier and its derivatives in three different solvent systems. These results are summarized in Table XIV.

3. EXPERIMENT III

Estrone-4⁻¹⁴C and estradiol-17β⁻⁴⁻¹⁴C were purified by TLC in system X just before use. A mixture of the two was injected into a hen who had been laying irregularly at the time of injection. The use of a mixture in this experiment was dictated by availability of labelled material at the time of the experiment. A mixture of aureomycin and streptomycin was dusted into the collection tube and urine was collected and stored as described in chapter II. The urines for the first two 24-hour periods after injection were processed separately.

At the time this experiment was undertaken, it was known that a negligible proportion of the injected radioactivity was excreted as glucosiduronates in the hen's urine (Mulay et al., 1968). Furthermore, appreciable amounts of the injected radioactivity appeared to be doubly conjugated, a conjugate which was partially hydrolyzed by incubation
at pH 4.5 although not by mere lowering of the pH. These observations made it evident that hydrolysis with  \( \beta \)-glucuronidase was not effective, while the partial hydrolysis of the conjugate on incubation suggested that sulfates might be present, since estrogen sulfates are known to be susceptible to prolonged pH changes. Recently, Mathur and Collins (1968) have shown that injected estrogens are excreted primarily as single and double sulfuric acid esters.

Accordingly, the procedure for processing urine was modified and the hydrolysis with 'Ketodase' was replaced by hydrolysis with 'Aryl sulfatase'. The procedure used for processing the urine samples is given below.

a. **Procedure for processing urine.**

Filtered urine (500 ml)

Urine buffered with 0.5 M acetate buffer by adding 4.1 g sod. acetate (anhydr.) and adjusting to pH 5 with g.a.a. 25 mg 'Aryl sulfatase' added to the buffered urine. Incubate for 24 hours at 37°C.

Extract with 3 x 1 vol. CHCl₃ — — — — — — — — — — — count

Incubate for another 24 hours at 37°C

Extract with 3 x 1 vol. CHCl₃ — — — — — — — — — — — count

Aqueous Residue

count
b. Results

The partition of radioactivity in various fractions obtained by processing the urine as described above were similar for each 24-hour period. Accordingly, the results for the two days were combined; they are summarized in Table XV.

The non-ketonic fraction subjected to TLC in system X and subsequently radioautographed. Fig. VI illustrates the clean separation of presumptive estradiol-17α from estradiol-17β. Zones corresponding to estradiol-17α and estradiol-17β were removed separately, eluted and counted. These results are also given in Table XV. The material corresponding to estradiol-17α was further purified by TLC in system X (double development).

A portion of the purified material was mixed with reference estradiol-17α and recrystallized from aqueous ethanol. Constant specific activity was attained after two crystallizations. These results are summarized in Table XVI.
TABLE XV

Experiment III: Recoveries of radioactivity in the urine and certain urinary fractions following the injection of a mixture of estrone-4-\(^{14}\)C and estradiol-17\(\beta\)-4-\(^{14}\)C into a laying hen.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Radioactivity as dpm x 10(^{-5})</th>
<th>percentage of urinary radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total injected</td>
<td>44.613</td>
<td>--</td>
</tr>
<tr>
<td>Urine*</td>
<td>13.949</td>
<td>100.0</td>
</tr>
<tr>
<td>CHCl(_3) extracts of enzyme treated urine</td>
<td>3.091</td>
<td>22.1</td>
</tr>
<tr>
<td>Residual aqueous phase</td>
<td>10.348</td>
<td>74.1</td>
</tr>
<tr>
<td>Pooled CHCl(_3) extracts used for Girard separation</td>
<td>3.003</td>
<td>21.5</td>
</tr>
<tr>
<td>Ketonic from Girard separation</td>
<td>1.400</td>
<td>10.0</td>
</tr>
<tr>
<td>Non-ketonic from Girard separation</td>
<td>0.746</td>
<td>5.9</td>
</tr>
<tr>
<td>Presumptive estradiol-17(\alpha)</td>
<td>0.1677</td>
<td>1.2</td>
</tr>
<tr>
<td>Presumptive estradiol-17(\beta)</td>
<td>0.2477</td>
<td>1.8</td>
</tr>
</tbody>
</table>

* The recovery of radioactivity in the urine as a percentage of injected material for the first 48 hours is 31.2%

Note: the ratio of estradiol-17\(\beta\) : estradiol-17\(\alpha\) for the laying hen was 1.5 : 1
TABLE XVI

Experiment III: Crystallizations of the urinary presumptive estradiol-17α and its diacetate to constant specific activity with carrier.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>dpm</th>
<th>mg</th>
<th>Specific activity dpm/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Estradiol-17α</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Original pool</td>
<td>87,881</td>
<td>25.80</td>
<td>3406</td>
</tr>
<tr>
<td>1st crystallization</td>
<td>64,314</td>
<td>19.14</td>
<td>3360</td>
</tr>
<tr>
<td>Mother liquor</td>
<td>18,067</td>
<td>5.60</td>
<td>3221</td>
</tr>
<tr>
<td>2nd crystallization</td>
<td>42,758</td>
<td>13.15</td>
<td>3252</td>
</tr>
<tr>
<td>Mother liquor</td>
<td>15,704</td>
<td>4.72</td>
<td>3327</td>
</tr>
<tr>
<td>3rd crystallization</td>
<td>24,620</td>
<td>7.55</td>
<td>3261</td>
</tr>
<tr>
<td>Mother liquor</td>
<td>13,341</td>
<td>4.10</td>
<td>3254</td>
</tr>
<tr>
<td><strong>Estradiol-17α diacetate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Theoretical value</td>
<td>--</td>
<td>--</td>
<td>2507</td>
</tr>
<tr>
<td>1st crystallization</td>
<td>43,605</td>
<td>18.52</td>
<td>2355</td>
</tr>
<tr>
<td>Mother liquor</td>
<td>8,147</td>
<td>4.70</td>
<td>1734</td>
</tr>
<tr>
<td>2nd crystallization</td>
<td>34,236</td>
<td>14.49</td>
<td>2363</td>
</tr>
<tr>
<td>Mother liquor</td>
<td>5,285</td>
<td>2.35</td>
<td>2249</td>
</tr>
<tr>
<td>3rd crystallization</td>
<td>20,064</td>
<td>8.56</td>
<td>2345</td>
</tr>
<tr>
<td>Mother liquor</td>
<td>9,381</td>
<td>3.98</td>
<td>2357</td>
</tr>
</tbody>
</table>
Figure VI: Experiment III. Radioautogram of chromatoplate of non-ketonic fraction of phenolic extract. Single development in system X. Circles indicate positions of the reference standards on pilot strips.

a = Estriol
b = 16-Epiestriol
c = 17-Epiestriol
d = Estradiol-17β
e = Estradiol-17α
f = Estrone

Note: The satisfactory separation of estradiols and the absence of estrone should be noted.
Figure VI: Experiment III. Radioautogram of chromatoplate of non-ketonic fraction of phenolic extract. Single development in system X. Circles indicate positions of the reference standards on pilot strips.

a = Estriol  
b = 16-Epiestriol  
c = 17-Epiestriol  
d = Estradiol-17β  
e = Estradiol-17α  
f = Estrone

Note: The satisfactory separation of estradiols and the absence of estrone should be noted.
TABLE XVII

Experiment III: Chromatographic mobilities of urinary presumptive estradiol-17α and its derivatives. In each case the mobility of the radioactive material coincided with the mobility of the carrier reference material. Numerical values as Rf.

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>A</th>
<th>C</th>
<th>X</th>
<th>Y₁</th>
<th>Y₂</th>
<th>O</th>
<th>Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Derivatives</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenol</td>
<td>0.60</td>
<td></td>
<td>0.42</td>
<td>0.23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diacetate</td>
<td></td>
<td>0.74</td>
<td></td>
<td></td>
<td>0.54</td>
<td>0.50</td>
<td>0.62</td>
</tr>
<tr>
<td>3-Methyl ether</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.14</td>
<td>0.22</td>
<td>0.40</td>
</tr>
<tr>
<td>Acetate of 3-</td>
<td></td>
<td>0.69</td>
<td></td>
<td></td>
<td>0.50</td>
<td>0.48</td>
<td>0.60</td>
</tr>
<tr>
<td>methyl ether</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The estradiol-17α crystals obtained after constant specific activity had been attained were subjected to acetylation. The acetylation product was recrystallized and constant specific activity was attained after three crystallizations. These results are also summarized in Table XVI.

A second portion of the presumptive radioactive estradiol-17α was mixed with approximately 1 mg of reference estradiol-17α and small amounts of this mixture were subjected to acetylation and methylation as described in chapter II. A portion of the methylation product was further acetylated. The phenol and its derivatives were chromatographed with appropriate reference materials in three different solvent systems. The identity of the radioactive estradiol-17α was confirmed by the complete congruence between the blackening on the radioautograms and the corresponding stain on the TLC plate. This has been illustrated in Fig. VII for the methyl ether of the presumptive estradiol-17α. The radioautogram of the chromatoplate and a photograph of the chromatoplate after spraying with colour reagent has been shown together to illustrate the complete correspondence in the shape and size of the blackening on the radioautogram and the colour spot on the plate. The Rf values of the phenol and its derivatives are summarized in Table XVII.

4. AD HOC EXPERIMENT

The results of experiments reported in sections 1, 2 and 3 of this chapter taken together fully satisfy the criteria for identification of radioactive metabolites; hence the results establish the presence of radioactive estradiol-17α in the urines of hens that had received
Figure VII: Experiment III.

1. Presumptive radioactive estradiol-17α mixed with reference estradiol-17α and methylated. The methylation product chromatographed in system 0.
   a = Reference estradiol-17α-3-methyl ether.
   b = Reference estradiol-17β-3-methyl ether.
   c = Methylation product of presumptive estradiol-17α plus reference estradiol-17α.

Note: i. The minor spots less polar than the 3-methyl ethers are believed to be due to the dimethyl ethers.
   ii. The minor spot more polar than the 3-methyl ethers is believed to be due to unreacted estradiol-17α.

2. Photograph of the radioautogram of chromatoplate shown in the 1st photograph.
   c = Blackening due the methylation product of presumptive radioactive estradiol-17α.

Note: The complete correspondence of shape and size of the blackening on the radioautogram and colour spots due to the methylation product of estradiol-17α (presumptive + Reference) shown as C on the 1st photograph.
Figure VII: Experiment III. Photographs illustrating the complete correspondence of the presumptive radioactive material with the reference estradiol-17α.
injections of estrone-4-$^{14}$C and/or estradiol-17β-4-$^{14}$C.

Before estradiol-17α could be considered as an *in vivo* urinary conversion product, it was necessary to establish that estradiol-17α was not formed in the urine after its excretion as a result of either microbiological action or the analytical procedures. The preliminary *ad hoc* experiments done in this connection (chapter III) established that (a) free estrogens, such as estrone-4-$^{14}$C, were converted to other radioactive compounds to a very small extent (2-4% of the radioactive estrone). (b) The radioactive products corresponding to estradiol-17α and estradiol-17β were shown to be neither of these two diols. (c) The conversion of estrone-4-$^{14}$C to other radioactive products was not brought about by the action of micro-organisms, although some of these were present in the urine which had been collected in the absence of antibiotics.

It was shown, therefore, that estrone-4-$^{14}$C when added to urine did not give rise to estradiol-17α. A similar experiment was done with estradiol-17β-4-$^{14}$C to exclude the possibility that radioactive estradiol-17α might be formed from estradiol-17β-4-$^{14}$C after excretion of urine.

Purified estradiol-17β-4-$^{14}$C was added to the collection tube attached to a hen that had not received any injection. Aureomycin and streptomycin mixture was dusted into the collection tube and the urine was collected and processed as described in chapter III. The chloroform extract residue was chromatographed in system X with appropriate reference estrogens on lateral strips. The zone corresponding to estradiol-17α was removed and eluted. The eluate was subjected to acetylation. The acetylation product was chromatographed in system Z with acetates of reference estradiol-17α, estradiol-17β, estrone and estriol as well as
reference phenols on lateral strips. These lateral strips were sprayed with the $\text{H}_2\text{SO}_4$-ethanol reagent. The regions corresponding to reference estrone acetate, estradiol-17β diacetate and estriol triacetate, as well as point of application were removed and counted.

These results showed that estradiol-17β-4-$^{14}$C formed a radioactive product which corresponded to reference estradiol-17α in its mobility, and which on acetylation and subsequent chromatography gave three spots on the radioautogram. The major amount of radioactivity remained at the point of application; a small amount of radioactivity had the same mobility as estrone acetate, whereas a very small amount of radioactivity had the same mobility as estradiol-17β diacetate. It is evident, therefore, that the bulk of the material which had the same mobility as estradiol-17α could not be acetylated, hence it could not have been estradiol-17α. These control experiments proved that radioactive estradiol-17α present in the urine was not formed by the action of micro-organisms after excretion or as a consequence of the analytical procedures.

C. DISCUSSION

The preliminary observations reported in experiment I (chapter IV section B,) were insufficient to identify radioactive estradiol-17α detected in the urine of a hen that had received an injection of estradiol-17β-4-$^{14}$C. The inclusion of a Girard separation in the procedure excluded the possibility of the presumptive estradiol-17α zone being either due to 16-ketoestrone or being partially contaminated by it.
It was further established that this zone was not estradiol-17β since it could be readily separated from estradiol-17β in system X, moreover their derivatives had mobilities different from one another in three different solvent systems and they corresponded with reference estradiol-17α and estradiol-17β and their respective derivatives.

The identity of presumptive estradiol-17α was established by recrystallization of the phenol to constant specific activity in experiments II and III and by recrystallization of the diacetate to constant specific activity in experiment III. This identification was further confirmed by the observations on the chromatographic mobilities of the phenol and its three chemical derivatives in all three experiments.

The percentage of radioactive material in the chloroform extracts in experiment III, which includes the free estrogens and the estrogens liberated by the sulfatase enzyme, was only 22.1% of the urinary radioactivity, a proportion which is considerably less than the corresponding value of 41.5% in experiment II. Other results by Mathur and Collins have shown that the estrogens in the occur as single and diconjugates of sulfates. This low percentage in experiment III is believed to be due to (a) relatively small amounts of free estrogens. [It has been observed that the percentage of free estrogens in the urine increases for samples stored for a longer duration of time before analysis. This aspect will be further elaborated upon in chapter VI of this thesis].

(b) There are reasons to believe that the sulfatase preparation used in experiment III was not satisfactory, because in other experiments in which a sulfatase preparation was utilized (chapter VI), it has rendered larger amounts of radioactivity extractable in chloroform. It is well known
that the presence of small amounts of moisture in sulfatase preparations during storage inactivates the enzyme.

The ad hoc experiments made in connection with experiments II and III excluded the possibility that estradiol-17α might have been formed as a result of microbiological action or analytical procedures after excretion of urine. The results established, therefore, that the hen is able to convert estrone and estradiol-17β to estradiol-17α in vivo. These conversions could be due to (a) metabolic conversions in the tissues of the hen or (b) microbiological conversions in the gut with reabsorption and subsequent excretion in the urine. The second possibility is inherent in experiments based on examination of urine for in vivo conversion products unless the experimental animals are germ-free. The first possibility, that the urinary estradiol-17α was formed by metabolic conversion in the tissues of the fowl, is the more likely, especially in view of the demonstration by Ozon and Breuer (1965) that chicken liver slices can convert estrone to estradiol-17α and estradiol-17β and that chicken liver contains 17α- and 17β- hydroxysteroid oxidoreductases. Renwick and Engel (1967) have recently achieved the separation and partial purification of 17α- and 17β- hydroxysteroid dehydrogenases from chicken liver. It may be concluded that the weight of the available evidence supports the view that the hen can form estradiol-17α in vivo and that estradiol-17α is to be included among the urinary steroid estrogens of the hen.

Attention should be drawn to the fact that the hen is the only animal in which estriol and its epimers have been shown to occur at the same time as estradiol-17α in the urine. In all other animals which
have been studied so far, either the estriols or estradiol-17α has been reported as present but not both. The only other animal in which there is some evidence for the simultaneous presence of estradiol-17α and estriol epimers in the urine is the dog, but much of the evidence on this species is contradictory. It may, therefore, be theorized that both pathways for metabolism of estrogens, namely, formation of estriols and formation of estradiol-17α are active in the hen. Metabolism of estrogens by either pathway, essentially, diminishes their biological potency and this process has been regarded by many as an inactivation process. Results presented in chapter VI, in which the distribution pattern of metabolites of estrogens in the laying hen have been compared, provides some support for the theory that both pathways are operative in the hen, since the amount of estradiol-17α increases in the laying hen when the production of estrogens is increased.
CHAPTER V

IN VIVO CONVERSION OF ESTRADIOL-17α-6,7-3H TO URINARY ESTRONE BY THE HEN
A. INTRODUCTION

Estradiol-17α was shown as an in vivo urinary conversion product of estrone and estradiol-17β in the hen (chapter IV). The interconversion of estrone and estradiol-17β in vivo was demonstrated by MacRae et al. (1959) and Ainsworth et al. (1962). Recently, Ozon and Breuer (1965) demonstrated the interconversion of estrone and estradiol-17α by chicken liver slices. Estradiol-17α has been shown to have a low estrogenic potency when tested in mammals and many groups of investigators have suggested that estradiol-17α represents the end conversion product of estrogen metabolism in some mammals such as the rabbit just as estriol has been considered to be the end product of estrogen metabolism in humans. In fact, Williams et al. (1968) have shown that when estradiol-17α is injected into rabbits it does not give rise to any other metabolites but is conjugated and excreted as such.

Although the reports by Ozon and Breuer (1965) and Renwick and Engel (1967) established the presence of the necessary enzymes in the chicken liver which could convert estradiol-17α to estrone, it was desirable to ascertain whether this transformation could occur in vivo.

B. EXPERIMENTAL AND RESULTS

1. EXPERIMENTAL

Estradiol-17α-6,7-3H was purified immediately before use by TLC in system X (double development). The purified material was pure as tested by TLC and appropriate examination of radioactivity on chromatoplate (see fig. VIII). The counting of the serial strips of silica gel
Figure VIII: Thin-layer chromatogram (system X) of estradiol-17α-6,7-3H used for injection. s.f. = solvent front. e = position of reference estradiol-17α.
on the plate was done as described in chapter II. The purified material was injected intramuscularly into a non-laying hen. The 24-hour samples were collected over six of the succeeding 7 days as described in chapter IV Experiment I. The recoveries of radioactivity in the urine were determined and the urine from the first three days of collection was processed as outlined in chapter IV, Experiment I, up to the extractions with chloroform. The residue from chloroform extracts was not subjected to a Girard separation, initially, but subjected to TLC in systems X with appropriate reference phenol estrogens on lateral strips. The distribution of radioactivity on the plate was determined as described before.

The major radioactive peak corresponding to estradiol-17α and estradiol-17β was subjected to Girard separation as described in chapter II.

2. RESULTS

The recoveries of injected estradiol-17α-6,7-3H in the 24-hour urine samples collected over six of the seven succeeding days after injection are summarized in Table XVIII.

The urinary recovery of the radioactivity (31.7%) was of the same order as those reported for injections of 16-ketoestradiol-17β (Ainsworth and Common, 1963); 16-epiestriol (Ainsworth et al., 1964), estradiol-17β and estrone (Mulay et al., 1968) under similar conditions.

The comparison of the percentage partitions of urinary radioactivity for the first three 24-hour urine samples did not reveal any differences as between these three samples. Accordingly, the data for the first three days were pooled and summarized in Table XIX. The loss of 22.9%
**TABLE XVIII**

Recoveries of injected radioactivity in urine, Estradiol-17α-6,7-3H (5.492 x 10^8 dpm) injected intramuscularly into a non-laying hen.

<table>
<thead>
<tr>
<th>Day</th>
<th>Urinary radioactivity as dpm</th>
<th>Urinary radioactivity as % of injected radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.65 x 10^8</td>
<td>29.9</td>
</tr>
<tr>
<td>2</td>
<td>6.45 x 10^6</td>
<td>1.2</td>
</tr>
<tr>
<td>3</td>
<td>2.37 x 10^6</td>
<td>0.4</td>
</tr>
<tr>
<td>4</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>5</td>
<td>0.78 x 10^6</td>
<td>0.14</td>
</tr>
<tr>
<td>6</td>
<td>0.21 x 10^6</td>
<td>0.04</td>
</tr>
<tr>
<td>7</td>
<td>0.09 x 10^6</td>
<td>0.02</td>
</tr>
<tr>
<td>Sum</td>
<td>1.74 x 10^8</td>
<td>31.7</td>
</tr>
</tbody>
</table>

* urine collection omitted on day 4
<table>
<thead>
<tr>
<th>Fractions</th>
<th>dpm</th>
<th>Percent total urinary radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total radioactivity in urine</td>
<td>$173.3 \times 10^6$</td>
<td>100</td>
</tr>
<tr>
<td>CHCl$_3$ extr. after 1$^{st}$ incubation</td>
<td>$26.1 \times 10^6$</td>
<td>--</td>
</tr>
<tr>
<td>&quot; &quot; &quot; 2nd &quot;</td>
<td>$3.6 \times 10^6$</td>
<td>--</td>
</tr>
<tr>
<td>&quot; &quot; &quot; 3rd &quot;</td>
<td>$3.2 \times 10^6$</td>
<td>--</td>
</tr>
<tr>
<td>Total CHCl$_3$ extracts</td>
<td>$32.9 \times 10^6$</td>
<td>19.0</td>
</tr>
<tr>
<td>Ethyl acetate extracts after CHCl$_3$ extracts</td>
<td>$20.6 \times 10^6$</td>
<td>11.9</td>
</tr>
<tr>
<td>Aqueous Residue</td>
<td>$80.0 \times 10^6$</td>
<td>46.2</td>
</tr>
<tr>
<td>Total radioactivity recovered in the urinary fractions</td>
<td>$133.5 \times 10^6$</td>
<td>77.1</td>
</tr>
</tbody>
</table>
of the radioactivity in the course of the experiments was high although not exceptional for experiments of this kind.

The residue from the pooled chloroform extracts was subjected to thin-layer chromatography and the distribution of radioactivity in the different zones was determined. The distribution of radioactivity on a typical chromatoplate is shown in Fig. IX. Three distinct peaks of radioactivity were observed. The mobility of the highest peak corresponded with the mobility of reference estradiol-17β and estradiol-17α. The mobility of the second highest peak corresponded with the mobility of reference estrone. The third peak, which was a minor one, was approximately in the same region as reference 16-epiestriol and 17-epiestriol. These zones were removed separately from this plate and other similar plates. The material from each zone was examined further as described below.

a. **Zone corresponding to reference estrone.**

The material in this zone accounted for 24% of the radioactivity on the chromatoplate. A portion of this material was mixed with reference estrone and recrystallized from aqueous ethanol. Constant specific activity was attained with the third crystallization. These results are summarized in Table XX. Another portion of the presumptive radioactive estrone was mixed with reference estrone and a part of this mixture was acetylated. Small samples of the phenol and its acetate were chromatographed in three different solvent systems. The sample was applied on the plate by a streaking technique. The position of the radioactive material was ascertained by counting series of silica gel strips (0.3 cm. wide) as described in chapter II. Only half of the
**TABLE XX**

Crystallization of radioactive urinary presumptive estrone to constant specific activity with reference estrone.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>dpm</th>
<th>mg</th>
<th>Specific activity dpm /mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; crystallization</td>
<td>197,600</td>
<td>17.5</td>
<td>11290</td>
</tr>
<tr>
<td>Mother liquor</td>
<td>96,200</td>
<td>6.2</td>
<td>15520</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; crystallization</td>
<td>154,900</td>
<td>15.3</td>
<td>10130</td>
</tr>
<tr>
<td>Mother liquor</td>
<td>21,000</td>
<td>2.0</td>
<td>10490</td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt; crystallization</td>
<td>102,700</td>
<td>10.2</td>
<td>10070</td>
</tr>
<tr>
<td>Mother liquor</td>
<td>37,500</td>
<td>3.7</td>
<td>10140</td>
</tr>
</tbody>
</table>
Figure IX: Distribution of radioactivity on thin layer chromatogram (system X) of CHCl₃ extracts of enzyme hydrolyzed urine. s.f. = solvent front

Positions of reference estrogens indicated as follows:

- a = Estriol
- b = 16-Epiestriol
- c = 17-Epiestriol
- d = Estradiol-17β
- e = Estradiol-17α
- f = Estrone

Note: 16-Ketoestrone is slightly less polar than estradiol-17α in system X.
material applied to the plate was subjected to counting; the remaining half was sprayed with either Folin-Ciocalten phenol reagent or H₂SO₄-ethanol reagent to visualize the reference estrone. The presumptive radioactive estrone did not give any colour when sprayed with the colour reagents, therefore the colouration was due to the reference carrier. Complete correspondence between the radioactive peak and the coloured spot was considered as proof of identity of presumptive estrone with the carrier. Fig. X illustrates congruence for the acetate of presumptive estrone in system O. The Rf values of the phenol and its acetate in three different systems are summarized in Table XXI.

The foregoing results demonstrated the identity of the urinary radioactive estrone and hence established the presence of radioactive estrone in the urine.

b. Zone corresponding to reference estradiol-17α, estradiol-17β, 16-ketoestrone and 16-ketoestradiol-17β.

The material in this zone (area between 5.5 cm. and 7.5 cm. in Fig.IX) accounted for 54.8% of the radioactivity on the plate. This region would include any 16-ketoestradiol-17β, if present, since this steroid is slightly more polar than estradiol-17β in system X. It was subjected to a Girard separation as described before. The ketonic fraction accounted for 19.9% of the radioactivity subjected to Girard separation, hence any 16-ketoestrone or 16-ketoestradiol-17β present could not have accounted for more than 19.9% of the radioactivity in this fraction or 10.1% of the total radioactivity on the chromatoplate. No further attempt was made to examine the materials in the ketonic fraction since the compounds likely to be present in this fraction are quite
TABLE XXI

Chromatographic mobilities of radioactive urinary estrone-^3H and its acetate.

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Solvent system</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Estrone-^3H</td>
<td>0.72</td>
</tr>
<tr>
<td>Estrone-^3H-acetate</td>
<td>--</td>
</tr>
</tbody>
</table>
Figure X: Acetate of presumptive radioactive estrone mixed with reference material and chromatographed in System O. Radioactive peak was located by counting serial strips (0.3 cm wide) of silica gel. The photograph shows complete correspondence between the radioactive peak and the colouration due to reference estrone acetate. Note: Only one radioactive peak is present indicating that the presumptive estrone fraction was pure.
Figure X: Acetate of presumptive radioactive estrone mixed with reference material and chromatographed in System O. Radioactive peak was located by counting serial strips (0.3 cm wide) of silica gel. The photograph shows complete correspondence between the radioactive peak and the colouration due to reference estrone acetate.

Note: Only one radioactive peak is present indicating that the presumptive estrone fraction was pure.
unstable; although 16-ketoestradiol-17β is relatively much more stable than 16-ketoestrone.

The non-ketonic fraction was chromatographed in system X (double development). The distribution of radioactivity on the chromatoplate is shown in Fig. XI. There was very sharp high peak of radioactivity precisely in the position of reference estradiol-17α with a shoulder in roughly the position of estradiol-17β. Further resolution of the diols was not attempted since the amount of radioactivity in the position corresponding to estradiol-17β was very small and its identification by crystallization or chromatography of the phenol and its derivatives was not feasible.

c. Zone corresponding to reference 16-epiestriol and 17-epiestriol.

The material in this zone accounted for 7.3% of the radioactivity on the chromatoplate. Appropriate amount of the radioactive material was applied to three plates between two spots containing a mixture of reference 16-epiestriol and 17-epiestriol. These plates were chromatographed in three different solvent systems. The reference estrogens were visualized and the regions corresponding to reference 16-epiestriol and 17-epiestriol were removed and counted. Both these zones were found to be radioactive in all the three solvent systems. These results provided evidence that the material in this zone contained two radioactive compounds which had the same mobilities as reference 16-epiestriol and 17-epiestriol in three different solvent systems. No derivatives could be prepared since the amount of radioactivity in this zone was insufficient.

These results did not provide sufficient proof for the identification
Figure XI: Distribution of radioactivity on thin layer chromatogram (system X, double development) of non-ketonic fraction from the major peak obtained by TLC of urinary CHCl₃ extracts (see Fig. IX).

s.f. = solvent front.

Positions of reference estrogens indicated as follows:-

a = estriol  
bc = 16-epiestriol  
c = 17-epiestriol  
d = estradiol-17β  
e = estradiol-17α  
f = estrone
of 16-epiestriol and 17-epiestriol, but they do provide some indication that the two cis-estriols were present.

3. AD HOC EXPERIMENTS

The foregoing results established the presence of radioactive conversion products of estradiol-17α-6,7-3H in the urine. The major conversion product was identified to be estrone and there was some indication for the presence of small amounts of estradiol-17β, 16-epiestriol and 17-epiestriol. To exclude the possibility that radioactive estrone and other conversion products were formed either by microbial action or by chemical changes after the excretion of urine, an ad hoc experiment was done in the following manner. Purified estradiol-17α-6,7-3H was added to the collection tube of a comparable hen that had not received any injection. The urine was collected and processed as outlined in chapter III Experiment I. The residue of the chloroform extract was chromatographed in system X with appropriate reference phenol estrogens. The distribution of radioactivity on the plate was determined as described before, and this is shown in Fig. XII. There was only one sharp radioactive peak corresponding to reference estradiol-17α and there was no indication of a radioactive peak in the region corresponding to reference estrone or any other estrogens. It was therefore concluded that radioactive estrone and other in vivo conversion products of estradiol-17α-6,7-3H in the hen were not formed by microbiological conversions or chemical changes after excretion.
Figure XII: Distribution of radioactivity on thin-layer chromatogram (system X) of CHCl₃ extract of urine control from uninjected bird (see text).

s.f. = solvent front

Positions of reference estrogens indicated as follows:

- a = estriol
- b = 16-epiestriol
- c = 17-epiestriol
- d = estradiol-17β
- e = estradiol-17α
- f = estrone
C. DISCUSSION

The recovery of radioactivity in the urine after injection of estradiol-17α-6,7-\(^{3}\text{H}\) and its excretion pattern over 6 days were comparable to similar recoveries with other injected estrogens. The percentage of radioactivity rendered extractable by chloroform after enzymatic hydrolysis accounted for 19% of the urinary radioactivity.

The major amount of the radioactivity remained in the aqueous fraction after enzymatic hydrolysis. It was evident, therefore, that the enzymatic hydrolysis was unsatisfactory. These experiments were done before it was known that estrogens in the hen's urine were present as monosulfates and disulfates. However, acid hydrolysis could not have been used since it is well known that acid hydrolysis destroys over 90% of estradiol-17α present in the urine. The results presented in this chapter are qualitative, therefore the amount of radioactivity in the chloroform extracts was sufficient for qualitative examinations of the radioactive conversion products.

Radioactive estrone was identified as a major urinary conversion product of injected estradiol-17α-6,7-\(^{3}\text{H}\) and there was some evidence for the presence of estradiol-17β, 16-epiestriol and 17-epiestriol. The possibility of formation of radioactive estrone by microbiological conversion of chemical changes after excretion was eliminated by ad hoc experimentation. This did not exclude the possibility of conversion by the action of intestinal micro-organism on estradiol-17α-6,7-\(^{3}\text{H}\) in the gut with reabsorption and subsequent excretion in the urine, but this possibility is inherent in \textit{in vivo} experiments unless germ free animals are used. The facts that (a) chicken liver slices convert
estradiol-17α to estrone (Ozon and Breuer, 1965) and that (b) chicken liver has been shown to contain the necessary enzymes to bring about such a conversion (Renwick and Engel, 1967) are consistent with the view that radioactive estrone found in the urine was mainly, if not entirely, the product of metabolic conversions in the tissues of the hen. Estrone has been shown to give rise to other in vivo conversion products (Ainsworth et al., 1962). The amount of metabolites tentatively identified as estradiol-17β and cis-estriols in chloroform extracts was considerably less than estrone. It can therefore be theorized that estradiol-17α is converted in vivo to other estrogens via estrone rather than by epimerization of estradiol-17α to estradiol-17β. The findings by Mathur and Common (1968b) confirm this hypothesis. Mathur and Common (1968b) have shown that when a mixture of 17β-estradiol-17α-3H and 17β-estradiol-4-14C is injected into a hen no tritium label was retained in the estradiol-17α, thus illustrating that estrone formation was an obligatory step in the formation of estradiol-17α from estradiol-17β.
CHAPTER VI

A STUDY OF SOME CONVERSION PRODUCTS OF ESTRONE-4-$^{14}$C IN THE LAYING AND NON-LAYING HEN WITH SPECIAL REFERENCE TO THE STATUS OF 16-KETOESTRONE AS A URINARY STEROID ESTROGEN.
A. INTRODUCTION

Early studies on the conversion of estrone-\(^{16}\)C in the laying hen indicated the presence of three major urinary products; these were estrone, estradiol-17\(\beta\) and a fraction tentatively identified as 16-ketoestrone. Both estrone and estradiol-17\(\beta\) have since been isolated and identified in the hen's urine. The tentative identification of 16-ketoestrone was based on the following evidence:— (a) lability of the presumptive 16-ketoestrone to acid hydrolysis; (b) chromatographic correspondence of the radioactive material with reference 16-ketoestrone on paper in chloroform-formamide system; (c) reduction of the presumptive 16-ketoestrone fraction with sodium borohydride yielded three radioactive products, the major reduction product having the same mobility as 16-epiestriol; and (d) failure to identify this zone as estradiol-17\(\alpha\).

The rigorous identification of estradiol-17\(\alpha\) as a urinary conversion product of injected estrone and estradiol-17\(\beta\) raises doubts about the earlier tentative identification of 16-ketoestrone as one of the major urinary conversion products of injected estrone. The reasons for these doubts may be summarized as follows:— (a) estradiol-17\(\alpha\) is also acid labile, therefore, if the presumptive 16-ketoestrone fraction in the earlier experiment was either completely or partially composed of estradiol-17\(\alpha\), it would also have been destroyed in an acid hydrolysis. Separation of estradiol-17\(\alpha\) and 16-ketoestrone is generally unsatisfactory (except in system X), therefore, it is quite possible that a resolution of these two compounds in the earlier experiment was incomplete. (b) Radioactive material which had been first subjected to radioautography for a long...
period of time is likely to contain several breakdown products, therefore the fact that the major reduction product had the same mobility as reference 16-epiestriol could not be considered as conclusive evidence for the identity of 16-ketoestrone.

In view of these doubts, a reexamination of the presumptive 16-ketoestrone fraction was undertaken with the view to identifying this steroid fraction more rigorously. It was felt that a Girard separation of the fraction would separate estradiol-17α from 16-ketoestrone, if present, and further identification could be attempted by reducing the ketonic fraction obtained by the Girard separation with sodium borohydride. The major radioactive product would be 16-epiestriol if 16-ketoestrone is, in fact, present and this product could be crystallized to constant specific activity and its derivatives also crystallized to constant specific activity. The extremely labile nature of 16-ketoestrone necessitated the use of an indirect method for its identification.

Chemical determinations of estrone (Common, Ainsworth, Hertelendy and Mathur, 1965), estradiol-17β (Mathur and Common, 1968a) and cis-estriol fraction (Mathur, Anastassiadis and Common, 1966) in 24-hour urines of laying and non-laying hens indicated that the excretion of these estrogens increased in the laying hen; furthermore, the amounts of estrone and estradiol-17β present were approximately of the same order in the laying hen, whereas the ratio of estradiol-17β to estrone was much higher in the non-laying hen. These observations strongly suggested that not only the amounts of urinary steroid estrogens but also their distribution patterns were different in the laying and non-laying hens. Accordingly,
a study of the distribution pattern of the urinary conversion products of estrone-4-$^{14}$C in a hen when she was laying and when she was not laying was undertaken to ascertain, whether there were any differences in the pattern of the in vivo conversion products of estrone as between laying and non-laying hens.

Since there is an increase in the excretion of estrone and the cis-estriols in the laying hen, it is reasonable to assume that larger amounts of 16-ketoestrone, if present, would be excreted by the laying hen. It was felt that any investigation of the status 16-ketoestrone as a urinary conversion product in the hen and its identification would be most fruitful in the laying hen.

B. EXPERIMENTAL

Purified estrone-4-$^{14}$C was injected into a hen, first, when she was not in lay and again, two months later, when she had come into full lay. A mixture of aureomycin and streptomycin was dusted into the collection tubes and the urine was collected for a 24-hour period after injection. The urines were stored at -15°C pending analysis. The urine collected from the hen when she was not in lay was processed 2 weeks after collection of the sample, whereas the urine collected from the hen when she was in lay was processed one day after collection. Both urine samples were processed as outlined below.
Filtered urine (500 ml)

extract with 2 x 1 vol. CHCl₃
count

hydrolysis of urine done with ' Aryl sulfatase' as described in Chapter II.

Extract with 2 x 1 vol. CHCl₃
count

Add 20 g of NaCl per 100 ml of urine

subject residue to TLC

and adjust to pH 1 with conc. HCl

and extract with equal volume of ethyl acetate immediately

organic phase

Aqueous residue

count

washed with 2 x 1/10 vol 4% NaHCO₃ sol. and 2 x 1/10 vol. water. Dry over sodium sulfate (anhydr.)
count

subject residue to TLC

The residues obtained from the free estrogen fraction, the enzyme-hydrolysed fraction and the solvolysis fraction were chromatographed in system X with appropriate reference phenolic estrogens and radioautograms of these plates were prepared. The zone corresponding to reference estradiol-17α was removed separately from each plate after determining
the proportions of radioactivity in different zones. This material was subjected to the Girard reaction as described before.

C. RESULTS AND DISCUSSION

The recoveries of radioactivity in the urine and other urinary fractions are given in Table XXII. The procedure used in processing the urine differed from those in the earlier experiment in one respect, namely, the inclusion of solvolysis; since estrogens are conjugated as sulfates in hen's urine, this step was included to free the maximum amount of the conjugated estrogens. The recovery of 20.4% of injected dose in the urine from the non-laying hen was lower than other similar recoveries but this low recovery is believed to be due to losses during collection. The recoveries of radioactivity in the free estrogen fraction were 8.9% and 3.2% for the non-laying and the laying hen, respectively. These differences were ascribed to the differences in the period of storage rather than to any differences in the degree of conjugation in the laying and the non-laying hens. The corresponding 'free estrogen' fraction in a similar partition study on the nature of estrogen conjugates in hen's urine (Mulay et al., 1968) ranged from 15 - 20% of the urinary radioactivity. In those studies the urine samples had been stored for a period of 4 weeks to 6 weeks after injection (not reported in the paper by Mulay et al., 1968). The fact that the 'free estrogen' fraction was as low as 3.2% in a urine sample stored for one day only and as high as 15 - 20% in urine samples stored for 4 - 6 weeks indicates that the free urinary estrogens represent a breakdown of estrogen conjugates during storage rather than an actual excretion of estrogens in the free form. In some
TABLE XXII

Recoveries of radioactivity in the urines and certain urinary fractions from a hen that had received estrone-4-$^{14}$C in the non-1aying phase and the laying phase.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>non-1aying</th>
<th>laying</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpm x 10^{-6}</td>
<td>% of urinary radioactivity</td>
</tr>
<tr>
<td>total injected</td>
<td>21.777</td>
<td>--</td>
</tr>
<tr>
<td>Urine</td>
<td>4.434</td>
<td>*</td>
</tr>
<tr>
<td>Radioactivity processed</td>
<td>4.064</td>
<td>100.0</td>
</tr>
<tr>
<td>Free estrogens</td>
<td>0.363</td>
<td>8.9</td>
</tr>
<tr>
<td>Estrogens liberated by sulfatase</td>
<td>1.074</td>
<td>26.4</td>
</tr>
<tr>
<td>Estrogens liberated by solvolysis</td>
<td>1.350</td>
<td>33.2</td>
</tr>
<tr>
<td>Aqueous Residue</td>
<td>1.187</td>
<td>29.2</td>
</tr>
<tr>
<td>Total recovery in the urinary fractions</td>
<td>97.7</td>
<td>96.6</td>
</tr>
</tbody>
</table>

* Recovery of radioactivity in the urine as a percentage of injected dose for the first 24 hour period was 20.8% for the hen in the non-1aying phase and 25.7% for the laying phase.
animals, such as the chimpanzee, large amounts of estrogens are excreted in an unconjugated form (Jirku and Layne, 1965). The present evidence, however, suggests that this is not true for the domestic fowl and that any free steroid estrogen present in the urine represents a breakdown of estrogen conjugates during storage.

The residues of the 'free estrogen' fraction, enzyme hydrolyzed fraction and the solvolysis fraction were chromatographed separately and the proportions of radioactivity in zones corresponding to reference phenolic estrogens were determined. The regions between these zones were also counted so that, in all, radioactivities in 11 fractions were determined. The results for the three urinary fractions were combined to get the distribution of radioactivity in the total phenolic steroid estrogen fraction and these are summarized in Table XXIII for the non-laying and the laying hen. The percentage of radioactivity was calculated on the basis of the total radioactivity in the phenolic steroid estrogen fraction.

Chromatography of the residues obtained from the 'free estrogens', enzyme hydrolyzed fraction and solvolysis fraction was done separately to see whether there were any major differences in the conversion products present in these fractions. Apart from the differences in the proportions of radioactivity in the estradiol-17β, estrone and estradiol-17α fractions, no other differences were observed. A greater proportion of the estrone present than of the estradiols was liberated by treatment with the 'Aryl sulfatase', and the subsequent solvolysis liberated a smaller proportion of the estrone and a greater proportion of the estradiols. These differences are explicable on the grounds that treatment with a phenol sulfatase would hydrolyze larger proportions of a single conjugate, such as estrone-3-sulfate, and only partially hydrolyze a double conjugate, such as estradiol-17β-3,
TABLE XXIII

Percent distribution of urinary conversion products of injected estrone-4-$^{14}$C for the laying and non-laying hen. All values as percent of the total phenolic steroid estrogens recovered from the urines.

<table>
<thead>
<tr>
<th>Chromatographic fractions</th>
<th>Major constituents</th>
<th>Non-laying</th>
<th>Laying</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Line of application</td>
<td>7.3</td>
<td>3.5</td>
</tr>
<tr>
<td>2</td>
<td>Trans-estriols</td>
<td>3.1</td>
<td>5.3</td>
</tr>
<tr>
<td>3</td>
<td>---</td>
<td>2.9</td>
<td>3.1</td>
</tr>
<tr>
<td>4</td>
<td>Cis-estriols</td>
<td>9.7</td>
<td>9.2</td>
</tr>
<tr>
<td>5</td>
<td>---</td>
<td>2.5</td>
<td>6.1</td>
</tr>
<tr>
<td>6</td>
<td>Unknown*</td>
<td>3.8</td>
<td>4.5</td>
</tr>
<tr>
<td>7</td>
<td>---</td>
<td>2.2</td>
<td>2.5</td>
</tr>
<tr>
<td>8</td>
<td>Estradiol-17β</td>
<td>49.0</td>
<td>17.9</td>
</tr>
<tr>
<td>9</td>
<td>Estradiol-17α and/or 16-ketoestrone**</td>
<td>7.7</td>
<td>19.9</td>
</tr>
<tr>
<td>10</td>
<td>---</td>
<td>1.4</td>
<td>1.9</td>
</tr>
<tr>
<td>11</td>
<td>Estrone</td>
<td>10.4</td>
<td>26.1</td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td>100.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

* The unknown fraction constituted a distinct band on the radioautogram and had the same Rf value as 16-ketoestradiol-17β in this system.

** The non-ketonic fraction consisting of estradiol-17α accounts for 90% of the radioactivity in this zone and is equivalent to 6.9% and 17.9% of the total phenolic steroid estrogens for the non-laying and laying hen respectively.
17-disulfate and its corresponding epimer; whereas solvolysis would completely liberate any partially hydrolyzed sulfate conjugate or a double conjugate. By combining the results from the three urinary fractions the distribution for the total urine was obtained, this distribution being more meaningful than the distributions in each individual fraction.

Zone 6 labelled as 'Unknown', constituted a single conversion product, since a distinct band was observed in this region on the radioautograms, as opposed to zones 3, 5, 7 and 9, in which there was no evidence for the presence of a distinct band. The Rf value of zone 6 in system X was very similar to the Rf value of reference 16-ketoestradiol-17β in system X. Identification of this band was not attempted.

Formal identification of estradiol-17β (zone 8) and estrone (zone 11) was not done, since these bands have already been identified by Ainsworth et al. (1962) and Hertelendy & Common (1965).

The presumptive estradiol-17α and/or 16-ketoestrone fraction (zone 9, tentatively identified as 16-ketoestrone by Ainsworth et al., 1962) obtained from each chromatoplate was subjected to Girard reaction separately. The proportions of ketonic and non-ketonic material in the presumptive estradiol-17α and/or 16-ketoestrone fractions are summarized in Table XXIV. Percentage values are based on the amount of radioactivity subjected to Girard separation.

The non-ketonic material accounted for 84.4% and 88.0% of the presumptive estradiol-17α and/or 16-ketoestrone fraction from the total phenolic steroid estrogens in the non-laying and the laying hen, respectively. The corresponding values for the ketonic fraction were 12.7%
TABLE XXIV

Proportions of non-ketonic and ketonic material in the presumptive estradiol-17α and/or 16-ketoestrone obtained by chromatography of urinary fractions. These fractions contain urinary conversion products of estrone-4-¹⁴C in the non-lying and the laying hen.

<table>
<thead>
<tr>
<th>Urinary fraction</th>
<th>Total</th>
<th>Non-ketonic</th>
<th>Ketonic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-lying hen</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free estrogens</td>
<td>1.742</td>
<td>1.581</td>
<td>0.158</td>
</tr>
<tr>
<td>(100 %)</td>
<td>(90.7 %)</td>
<td>(9.0 %)</td>
<td></td>
</tr>
<tr>
<td>Material liberated by 'Aryl sulfatase'</td>
<td>2.547</td>
<td>2.288</td>
<td>0.230</td>
</tr>
<tr>
<td>(100 %)</td>
<td>(89.8 %)</td>
<td>(9.0 %)</td>
<td></td>
</tr>
<tr>
<td>Material liberated by solvolysis</td>
<td>4.244</td>
<td>3.327</td>
<td>0.696</td>
</tr>
<tr>
<td>(100 %)</td>
<td>(78.3 %)</td>
<td>(16.3 %)</td>
<td></td>
</tr>
<tr>
<td>TOTAL</td>
<td>8.533</td>
<td>7.196</td>
<td>1.084</td>
</tr>
<tr>
<td>(100 %)</td>
<td>(84.4 %)</td>
<td>(12.7 %)</td>
<td></td>
</tr>
<tr>
<td><strong>Laying hen</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Material liberated by 'Aryl sulfatase'</td>
<td>3.196</td>
<td>2.791</td>
<td>0.283</td>
</tr>
<tr>
<td>(100 %)</td>
<td>(88.0 %)</td>
<td>(8.9 %)</td>
<td></td>
</tr>
<tr>
<td>Material liberated by solvolysis</td>
<td>4.737</td>
<td>2.791</td>
<td>0.537</td>
</tr>
<tr>
<td>(100 %)</td>
<td>(87.8 %)</td>
<td>(11.3 %)</td>
<td></td>
</tr>
<tr>
<td>TOTAL</td>
<td>7.906</td>
<td>6.950</td>
<td>0.820</td>
</tr>
<tr>
<td>(100 %)</td>
<td>(87.9 %)</td>
<td>(10.4 %)</td>
<td></td>
</tr>
</tbody>
</table>

* The activity in the presumptive estradiol-17α and/or 16-ketoestrone from the free estrogen fraction was too low, therefore, no Girard reaction was done
and 10.4% respectively. Losses in the course of Girard separations were negligible.

These results indicate that the acid-labile urinary steroid fraction that was tentatively identified as 16-ketoestrone by Ainsworth et al. (1962) comprises at least 86% non-ketonic material and only 10-13% ketonic material. The non-ketonic material had the same mobilities as reference estradiol-17\(\alpha\) in three different solvent systems. This estrogen had already been identified as a urinary conversion product of estrone and estradiol-17\(\beta\), therefore it may be concluded that the fraction tentatively identified by Ainsworth et al. (1962) as 16-ketoestrone was, in fact, at least 90% estradiol-17\(\alpha\), and that 16-ketoestrone, if present, was a relatively minor urinary conversion product. A rigorous identification of the ketonic material could not be attempted since the amounts of the ketonic material were insufficient. These results do not exclude the presence of 16-ketoestrone amongst the urinary conversion products but they do demonstrate that 16-ketoestrone, if present, is a minor conversion product, whereas estradiol-17\(\alpha\) is a major urinary conversion product in the laying and non-laying hen.

From the quantitative standpoint the three most important steroid estrogens in the non-laying hen were first, estradiol-17\(\beta\) (zone 8 = 49%); second, estrone (zone 11 = 10.4%) and third, presumptive estradiol-17\(\alpha\) and/or 16-ketoestrone (zone 9 = 7.7%). Since at least 90% of this fraction comprised of estradiol-17\(\alpha\), therefore at least 6.9% of the radioactivity in zone 9 was due to estradiol-17\(\alpha\). The combined cis-estriol fraction (zone 4 = 9.7%) was greater than the estradiol-17\(\alpha\) fraction (6.9%) but when measured separately both 16-epiestriol and 17-epiestriol
were less in amount than estradiol-17α.

The most important urinary estrogens in the laying hen were, first, estrone (zone 11 = 26.1%); second estradiol-17β (zone 8 = 17.9%) or estradiol-17α which comprised 90% of the presumptive estradiol-17α and/or 16-ketoestrone (zone 9 = 19.9%) and was equivalent to 17.9% of the radioactivity.

The patterns of excretion of the three major urinary conversion products of injected estrone-4-14C were different as between the non-laying and laying phase. These differences are best illustrated by Fig. XIII, in which a comparison of the percentage distribution of radioactivity in various fractions for the laying and the non-laying phase is made by plotting the percentages from Table XIX.

For the non-laying phase, the ratio of estrone: estradiol-17β was 1:4.7, whereas for the laying phase this ratio was 1:0.69. These ratios are in general agreement with similar ratios obtained by direct determination of estrone and estradiol-17β by methods based on the Ittrich-Kober colour reaction. The ratio of estradiol-17β: estradiol-17α was 7.1:1 for the non-laying state and 1:1 for the laying state.

Similar ratios were obtained from experiments II and III described in chapter IV. The ratio of estradiol-17β: estradiol-17α for non-laying state was 8.7:1 (see Table XV note) and 1.5:1 for the laying state (see Table XVII foot note). It should be noted that these ratios from experiments II and III (Chapter IV) were from two different hens. The ratios of estradiol-17β: estrone could not be calculated for the hens used in experiments II and III since Girard reaction was done on the total phenolic fraction prior to separation of urinary conversion products by
chromatography.

The changes in the relative proportions of the other estrogens were slight, as can be seen in Fig. XIII, so that the decrease in the proportion of estradiol-17\(\beta\) as the hen moved from the non-laying state to the laying state was approximately equivalent to the increase in the proportion of estradiol-17\(\alpha\) and estrone (see Fig. XIV). The proportion of cis-estriol fraction in these experiments did not change appreciably, in fact, there was a slight decrease as the hen moved from the non-laying state to the laying state. The evidence presented by Mathur et al. (1966) indicates that there is increase in the amount of cis-estriols excreted by the laying hen. This apparent lack of increase could be due to the fact that no purification of the cis-estriol fraction was done before determining the ratios and the blackening on the radioautograms indicated the presence of radioactive impurities which may obscure any real increase in the proportion of cis-estriols.

It has been established that the laying hen excretes more steroid estrogens than the non-laying hen. The results now present show that the distribution pattern of urinary steroid estrogens also differs as between the laying and non-laying hen. Moreover, these results strongly suggest that the in vivo metabolism of the steroid estrogens in the non-laying hen differs from that in the laying hen such that the pathway leading to formation of estradiol-17\(\alpha\) is relatively more active in the laying hen. It may be theorized that the hen handles the increased amounts of estrogens in a qualitatively different manner. In this connection, it should be noted that the ratio of estradiol-17\(\alpha\) to estrone in the non-laying hen is 0.66, whereas the corresponding ratio in the laying hen is
Figure XIII: Percentage distribution of urinary phenolic conversion product of injected estrogens. Values for non-laying state as clear columns. Values for the laying state as solid black columns.

Zone 4: 16-Epiestriol plus 17-epiestriol
Zone 8: Estradiol-17β
Zone 9: Approximately 90 percent estradiol-17α
Zone 11: Estrone
0.68. These results, strongly suggest that the 17β-hydroxysteroid dehydrogenase rather than the 17α-hydroxysteroid dehydrogenase is activated when the hen moves from the non-laying to the laying stage. Further experimentation will be necessary to evaluate the changes in the metabolic equilibria between the laying and non-laying hens.
SUMMARY

1. *Ad hoc* preliminary experiments have been performed to check whether radioactive steroids used in *in vivo* studies and their metabolites were converted to other radioactive compounds during collection and storage and if these changes were effected by either micro-organisms or by chemical changes.

These experiments have revealed the following:-

a. A small proportion (2.2%) of radioactive estrone is converted to other radioactive products.

b. Although bacteria are present in urine samples collected in the absence of an antibiotic in the collection tubes, these are not responsible for the conversion of radioactive estrone to other radioactive compounds.

c. Aureomycin and streptomycin are most suitable among those tested, for controlling the growth of bacteria in the collection tubes in so far as these antibiotics do not interfere in the thin-layer chromatography of urine extracts.

d. Radioactive estrone forms two conversion products, among others, which have the same mobility as estradiol-17β and estradiol-17α in three different solvent systems but these products cannot be acetylated, thus indicating that neither of these two products is estradiol-17β nor estradiol-17α.

e. Radioactive estradiol-17β forms a conversion product among others, which has the same mobility as reference estradiol-17α but the bulk of this product cannot be acetylated, thus indicating that no estradiol-17α is formed from estradiol-17β during collection and storage of urine.
f. Radioactive estradiol-17α does not undergo any changes during collection and storage of urine.

2. A modification of the method for counting silica gel by suspension in thixotropic gel (Snyder & Stevens, 1962) has been described. This simple modification eliminates difficulties experienced previously in pouring exact volumes of thixotropic gel.

3. Estradiol-17α has been shown to be a normal metabolite of radioactive estrone and estradiol-17β in the laying and non-laying hen. Identification has been based on chromatographic correspondence of the phenol and its three derivatives with reference material in three different solvent systems and the attainment of constant specific activity of the phenol and its acetate with reference material.

4. Estrone has been identified as a metabolite of estradiol-17α-6,7-3H. Identification has been based on the chromatographic correspondence of the phenol and its acetate with reference material in three different systems and the attainment of constant specific activity of the phenol with reference estrone. Some evidence has been provided for the presence of estradiol-17β, 16-epiestriol and 17-epiestriol. Presence of ketonic material other than estrone has also been noted.

5. The distribution pattern of the in vivo conversion product of estrone-4-14C in a hen during the non-laying and the laying phase has been studied and these studies have revealed the following:-

a. Very little 'free estrogens', if any, are excreted by the hen. The higher amounts of 'free estrogens' observed in these and previously reported experiments (Mulay et al., 1968) are due to breakdown of conjugates during storage and this proportion increases with increased period of storage.
b. 16-Ketoestrone is not, as hitherto suspected a major urinary conversion product in the non-lying as well as the laying hen.

c. Estradiol-17α is a major urinary conversion product in the non-lying as well as the laying hen. It is the third major estrogen in the non-lying hen and the second major estrogen in the laying hen.

d. The decrease in the radioactivity in the estradiol-17β fraction as the hen moves from the non-lying state to the laying state is equivalent to the increase in the radioactivity in the estrone and estradiol-17α fractions. The ratios of the three major estrogens, estradiol-17β : estradiol-17α : estrone in the non-lying hen and the laying hen are 7.1 : 1 : 1.5 and 1 : 1 : 1.5 respectively.
CLAIMS TO ORIGINAL RESEARCH

1. The demonstration that the hen can convert $^{14}$C-labelled estrone and estradiol-17β to estradiol-17α among other urinary conversion products. (Identification of estradiol-17α was based on the chromatographic correspondence of the phenol and its three derivatives with reference material and the attainment of constant specific activity of the phenol and its acetate with appropriate reference materials.)

2. The demonstration that the urinary radioactive estradiol-17α thus produced constitutes the third major urinary conversion product in the non-laying hen and the second major conversion product in the laying hen.

3. The demonstration that 16-ketoestrone is not, as hitherto suspected, a major urinary steroid estrogen in hen's urine.

4. The demonstration that the hen can convert injected radioactive estradiol-17α into urinary radioactive estrone and less certainly other radioactive steroid estrogens. (Identification of estrone was based on the chromatographic correspondence of the phenol and its acetate with reference material in three different solvent systems and the attainment of constant specific activity with appropriate reference material.)

5. The demonstration that the distribution of the urinary products of injected tracer doses of estrone in non-laying hen differs from that in they laying hen. The decrease in estradiol-17β fraction as the hen moves from the non-laying stage to the laying stage is equivalent to the increase in the estrone and estradiol-17α fractions. Thus, the ratio of the three major urinary steroid estrogens, estradiol-17β : estradiol-17α :estrone was 7.1 : 1 : 1.5 in the non-laying hen, whereas this ratio was 1 : 1 :1.5 in the laying hen.
The foregoing contributions represent, to the best of the candidate's knowledge, the first reported evidence for the presence of estradiol-17α in the urine of any avian species and the first report on the in vivo conversion of estradiol-17α in any avian species.
PUBLICATIONS BY THE CANDIDATE

1. Mulay, S., and Common, R.H.
   Seventh International Congress of Biochemistry, Abstracts IV,
   p. 748, Tokyo, 1967.

2. Mulay, S., Carter, A.L. and Common, R.H.

3. Mulay, S. and Common, R.H.

4. Mulay, S., Henneberry, G.O. and Common, R.H.
BIBLIOGRAPHY

Studies on Oestrogen Excretion in Human Bile.

Gas Chromatographic Studies on Oestrogen in the Bile of Pregnant
Women.

On the Nature and Metabolism of Oestrogens in the Domestic Fowl.

Surgical Procedure for Exteriorizing of the Ureteral Openings of
the Hen.
Poultry Sci., 44: 1561-1564.

Crystalline Oestrone Isolated from Urine of Laying Hen.

Urinary and Fecal Conversion Products of 16-Oxoestradiol-17β-16-C\(^{14}\)
in the Domestic Fowl.

A Chromatographic Study of Some Conversion Products of Estrone-16-C\(^{14}\)
in the Urine and Faeces of the Laying Hen.

Urinary Conversion Products of 16-Epiestriol-16-C\(^{14}\) in the Domestic
Fowl.

Embryologie Chimique - Sécrétion d'Oestrone et l'Oestradiol par le
Testicule Féminisé de l'Embryon de Poulet.

Secretion of Estrone and Estradiol by In Vitro - Cultured Ovaries from
Duck Embryos.
A Simple Method for Analyzing Complicated Absorption Curves of Use in the Colorimetric Determinations of Urinary Steroids.  


The Metabolism of Estrone-16-C<sup>14</sup> in Bovine Blood.  

Methylation of 2-Hydroxyestradiol-17β to 2-Methoxyestrone in the Human.  

The Conversion of 2-Hydroxyestradiol-17β to 2-Hydroxy and 2-Methoxy Metabolites in Human Urine.  

Definitive Identification of Microquantities of Radioactive Steroids by Recrystallization to Constant Specific Activity.  

Estrogen Metabolism in the Male Dog.  
Uptake and Disappearance of Specific Radioactive Estrogens in Tissues and Plasma Following Estrone-6,7-<sup>3</sup>H Administration. Identification of Estriol-16α,17α in Tissues and Urine.  
Endocrinology, 82: 500-510.

Bauld, W.S., 1954.  
Some Errors in the Colorimetric Estimation of Oestriol, Oestrone and Oestradiol by Kober Reaction.  

Baulieu, E.E., Corpéchot, C., Dray, F., Emilozzi, R., Lebeau, M.C. 
An Adrenal-Secreted 'Androgen': Dehydroisoandrosterone Sulfate. Its Metabolism and a tentative Generalization on the Metabolism of Other Steroid Conjugates in Man.  

Beall, D., 1939.  
Isolation of Estrone from the Adrenal Gland.  
Nature, 144: 76.
Beall, D., 1940.
The Isolation of α-Oestradiol and Oestrone from Horse Testes.

Excretion of Estrogen Metabolites by Humans. I. The Fate of Small Doses of Estrone and Estradiol-17β.
J. Biol. Chem., 214: 335-349.

Excretion of Estrogen Metabolites by Humans. II. The Fate of Large Doses of Estradiol-17β After Intramuscular and Oral Administration.


Gel Filtration of Conjugated Urinary Oestrogens and its Application in Clinical Assays.
Acta. Endocr., Suppl. 79.

Stoffwechsel von 16-Oxo-17β-Ostradiol beim menschen.

Berthold, A.A., 1849.
Cited from Burrows, 1949, p.177


The Metabolism of Natural Estrogens.

Biochemical Aspects of Oestrogen Metabolism.

Formation of Oestriol-3,16β,17α by Liver Tissue in vitro.
Stoffwechsel von Oestron, Oestradiol-17α und Oestradiol in der Kaninchenleber.


Studies on the Metabolism of 17α-Estradiol in Man.

Metabolism of Oestrone and Oestradiol-17β in Human Liver Tissue.
Nature, 212: 76.

Metabolism of 16β-Hydroxyoestrone in vitro.

Stoffwechsel von 16-Keto Oestron in Menschlichen Geweben.

Zwischenstoffwechsel der 16,17-Substituierten Östrogene in der Kaninchenleber.

Konfiguration, Biogenese und Stoffwechsel von 6α- und 6β-
Hydroxylierten Phenolischen Steroiden.

Reduktion von 16α-Hydroxy-oestron und 16-Keto-Oestradiol-(17β)
durch Menschliches Lebergewebe in vitro.

Stoffwechsel der Östrogene in Leberschnitten der Ratte.


The Metabolic Reduction of 16α-Hydroxyoestrone to Oestriol in Man.

The Effect of Nutritional Status and Thyroid Function on Metabolism of Estradiol.

Urinary Estrogen Excretion in Humans.

Browne, J.S.L., 1930.
Cited from Diczfalusy and Lauritzen, 1961, p. 53.

Metabolism, Excretion and Retention of Carbon-14-labelled Estrone in Immature Mice.

'Biological Actions of the Sex Hormones.'

Hydrolysis of Ketosteroid Hydrogen Sulfates by Solvolysis Procedure.

The Isolation of 6 'α'-Hydroxyoestradiol-17β from the Follicular Fluid of the Mare.
J. Endocr., 20: i.

Butenandt, A., 1929.
Progynon, a Crystalline Female Sexual Hormone.
Naturwiss., 17: 879.

6α-Hydroxylation in Human placenta Perfusion Steroids, 6: 307.

Stoffwechsel und Aromatisierung von 7-Substituierten C-19-Steroiden in der Placenta.


Isolation and Identification of 16-oxo-17β-Estradiol in Human Placenta.

Dingemanse, E., Laqueur, E. and Mühlbock, O., 1938.
Chemical Identification of Oestrone in Male Urine.

Cited from Breuer, 1962.

Surgical Technique for the Exteriorization of the Ureters of the Chicken.

The Preparation of Theeolol.

Metabolism of the Estrogens.

Folliculine from Urine of Pregnant Women.

Metabolism of the Steroid Hormones: The Excretion of Estrone After the Administration of Estrone and α-Estradiol to Rhesus Monkeys.


Endocrinology, 61: 113-114.

The Application of Countercurrent Distribution to the Separation and Characterization of Urinary Estrogens.
The Estimation of Urinary Metabolites of Administered Estradiol-17β-16-C\textsuperscript{14}.

Studies on Oestrogens in Cattle. Oestrogen Conversions in Different Tissues Grown In Vitro.

Fish, W.R. and Dorfman, R.I., 1942.
Metabolism of Steroid Hormones. II The Conversion of α-Estradiol to Estrone and β-Estradiol by Ovariectomized - Hysterectomized Rabbit.

Role of 2-Hydroxyestrone in Estrogen Metabolism.

2-Methoxyestradiol: A New Metabolite in Man.

Oxidative Metabolism of Estradiol.

2-Hydroxyestrone: A New Metabolite of Estradiol in Man.

Pathway and Stereochemistry of Formation of Estriols in Man.
Biochemistry, 5: 1789-1794.

Effect of Thyroid on Hydroxylation of Estrogen in Man.

Metabolism of Estriol-17α-\textsuperscript{3}H in Man.
Steroids, 11: 337-346.

Further Studies on the Metabolism of Oestradiol in Man.

Metabolism of 17β-Estradiol-4-\textsuperscript{14}C in a Non-pregnant Rhesus Monkey.
The Conversion of Dehydroepiandrosterone-7α-3H to Oestrogens by Corpora-lutea of Early Human Pregnancy.

Folin, O. and Ciocalteu, V., 1927,
On Tyrosine and Tryptophane Determinations of Proteins.

Frandsen, V.A., 1959.
The Demonstration of 2-Methoxyestrone and 2-Methoxyestradiol in Human Pregnancy Urine.

Formation of Egg in Domestic Chicken.

Sur Une Novelle Hormone Sexuale Cristallisée.


A Method for the Quantitative Fractionation of Mixture of 2-Methoxyoestrone, Oestrone, Ring D – Ketolic Oestrogens, Oestradiol-17β, 16-epiOestriol and Oestriol by Partition Chromatography and Girard Reaction.

Identification of Estrogens in Human Testis.

Characterization of Estrogens in the Bovine.
Endocrinology, 64: 707-711.

The Occurrence and Biological Significance of Steroids in the Lower Vertebrates.
A Review.

Biosynthesis of Estrogens.
Endocrinology, 71: 920-925.
Gustavson, R.G., 1931.
The Occurrence of Estrin in the Feces of Hen.

Metabolism of 17β-Oestradiol-4-14C in Early Infancy.

Etude en Culture Organotypique In Vitro du Métabolisme de la Déhydroépiandrostérone et de la Testostérone Radioactives, par les Gonades Normales et Intersexuées de l'Embryon de Poulet.

Häussler, E.P., 1934.
Über das Vorkommen von α-Follikelhormon (3-oxy-17-keto-1,3,5, Ostratien) im Hengsturin.

Metabolism of Tritiated Estradiol in Laying Hen.

Steric Considerations in the Enzymatic Course of Hydroxylation of Steroids.

The Metabolism of the Estrogens.

The Isolation of Δ-5,7,9-Estratrienol-3-one-17 from the Urine of Pregnant Mares.

Steroids: IV The Fate in Man of Injected α-Estradiol.

The Metabolism of Estrogens.

Steroids: V α-Estradiol and Progesterone Metabolism.

Heller, C.G., 1940.
Metabolism of Estrogens. The Effect of Liver and Uterus upon Estrone, Estradiol and Estriol.
Endocrinology, 26: 619-630.
Hertelendy, F. and Common, R.H.  
Isolation of Crystalline 16-Epiestriol from Hen Urine.  

In Vivo Conversion Products of Estradiol-16-C14-17β in the Hen.  
Poultry Sci., 44: 1379.

Isolation of Estradiol-17β from Hen's Urine and Its Characterization  
as the Crystalline 3-Methyl Ether.  

Hirschlmann, H. and Wintersteiner, O., 1938.  
The Isolation of Estrogenic Diols from the Urine of Pregnant Mares.  

Ring D -α-Ketols as Metabolic Products of Estrogen in the Non-pregnant  
Human Subject.  

2-Methoxyestrone as an Estrogen Metabolite in the Human Subject.  

Specific Activities of Seven Urinary Metabolites of Estradiol-  
17β-6,7-3H in Pregnant Women.  

Peripheral Interconversions of Phenolic Steroids in the Human.  

The Relative Estrogenic Activity of Compounds Related to Estriol.  
Endocrinology, 41: 12-16.

The Isolation of α-Dihydrotheeolin from Human Placenta.  

Huffman, M.N., MacCorquodaile, D.W., Thayer, S.A., Doisy, E.A., Smith, G.V.  
and Smith, O.W., 1940.  
The Isolation of α-Dihydrotheeolin from Human Pregnancy Urine.  

Metabolism of Estrone-16-C14 in the Luteal Phase Bovine.  
Report at the Fifth Symposium of Animal Reproduction, University of  
Tennessee, Knoxville.  


Biogenesis of 11β-Hydroxyestrone and 16α-Hydroxyestrone by Adrenal Tissue.

15α-Hydroxylierung von Östron in der Nebenniere.

Cited from Chem. Abstr., 66: 282m.

Isolierung von 15α-Hydroxy-Oestradiol-(17β) aus dem Urin Schwangerer Frauen.

Biogenese von 6α-Hydroxy-Östron in der Nebenniere.

Biogenesis of 7α-Hydroxyestrone by Adrenal Tissue.

Isolation and Identification of 15α-Hydroxyestrone from Urine of Pregnant Women.

Formation of 15α-Hydroxyestradiol-17β and 18-Hydroxyestrone by Human Adrenal Tissue.
J. Endocr., 33: 529.

Isolation and Identification of 15α-Hydroxyestrone and 15β-Hydroxyestradiol-17β from Urine of Pregnant Women.
Steroids, 8: 403-414.

The Isolation of 6α-Hydroxyestrone from Urine of Pregnant Women.

14α-Hydroxyestrone a New Estrogen Metabolite.
2-Methoxyestrone a Metabolite of Estradiol-17β in the Human.  

2-Methoxyestrone a New Metabolite of Estradiol-17β in Man.  

Urinary Estrogens of the Dog.  

Thermo-Microscopic and Spectrophotometric Determination of Steroid Hormones.  
Microchem. J., 9: 105-133.

Isolation of Estrone, 17β-Estradiol and Estriol from Female Human Urine.  
Steroids, 2: 297-318.

Human Placental Estradiol-17β Dehydrogenase, 1. Concentration, Characterization and Assay.  

Layne, D.S., 1957.  
On the Nature of Gonadal Hormones of the Domestic Fowl  

Identification in Rabbit Urine of 3-Glucuronoside-17-N-Acetyl Glucosaminide of 17α-Estradiol.  
Endocrinology, 76: 600-603.

Isolation of 16β-Hydroxyoestrone from the Urine of Pregnant Women.  

The Isolation of 16β-Hydroxyestrone and 16-Ketoestradiol-17β from the Urine of Pregnant Women.  

The Isolation from Rabbit Urine of a Conjugate of 17α-Estradiol with N-Acetyl-glucosamine.  
Presence of Oestrone, Oestradiol and Oestriol in the Extracts of Ovaries of Laying Hens.

Charakterisierung und Kinetic einer Mikrosomalen 17β-Hydroxysteroid-Oxidoreductase der Menschlichen Placenta.

Stoffwechsel von 16-Oxo-Oestrone in Zellfractionen der Rattenleber.

Metabolism of 16-Oxoestriol in Cellular Fraction of Human Placenta.

Levin, L., 1945a.
The Isolation of α-Estradiol from the Urine of Stallions.
J. Biol. Chem., 158: 725-726.

Levin, L., 1945b.
The Fecal Excretion of Estrogens by Pregnant Cows.

Interconversions of 16-Oxygenated Estrogens. II. The Metabolism of 16-Ketoestriol-17β-16-C14 in Man.

Levitz, M., Spitzer, J.R. and Twombly, G.H., 1956.
The Conversion of Estradiol-17β-16-C14 to 16-Ketoestradiol-17β in Man.


Transfer of Estriol and Estriol Conjugates Across Human Placenta Perfused In Situ at Mid-pregnancy.

The Monohydroxylation of 17β-Estradiol at 15α-, 16α-, and 16β- positions by Bovine Adrenal Perfusion.


Estrogen Metabolism In Vitro in the Presence of Mammalian
Enythrocytes. A Comparative Study.

Isolation and Identification of 11-Dehydro-17α-Estradiol; A New
Type of Urinary Steroid in the Urine of Pregnant Women.

The Isolation of the Principal Estrogenic Substance Liquor Folliculi.

MacRae, H.F. and Common, R.H., 1960.
Formation of 16-Epiestriol from Estradiol-17β in the Laying Hen.

Formation In Vivo of 16-Epiestriol and 16-Keto-Estradiol from Estriol
by the Laying Hen and Occurrence of Equol in Hens' Urine and Faeces.

MacRae, H.F., Layne, D.S. and Common, R.H., 1959.
Formation of Oestrone, Oestriol and an Unidentified Steroid from
Oestradiol in the Laying Hen.
Poultry Sci., 38: 684-687.

Isolation of Crystalline Estradiol-17β from the Droppings of
Laying Hens.

Mahesh, V.B. and Herrmann, W., 1963.
Isolation of Estrone and 11β-Hydroxyestrone from a Feminizing
Adrenal Carcinoma.
Steroids, 1: 51-61.

Marker, R.E., 1939.
Sterols LXI: The Steroidal Content of Steer's Urine.

Sterols XLII: The Isolation of Oestranediols from Human Non-pregnancy
Urine.

Marrian, G.F., 1930.
The Chemistry of Oestrin III: An Improved Method of Preparation and
Isolation of Active Crystalline Material.
The Biochemistry of Oestrogenic Hormones.

Some Recent Advances in Estrogen Biochemistry.

The Isolation of a Fourth Kober Chromogen from the Urine of Pregnant Women.

The Isolation of 16-Epioestriol from the Urine of Pregnant Women.

16α-Hydroxyoestrone in the Urine of Pregnant Women.

A Protein Conjugated Metabolite of Estrone-4-14C
Cited from Chem. Abstr., 67: 29525u

Experiments on Organ Extracts.
XXIV The Hormonal Active Principle of a Feminizing Testicular Tumor.

Steroid Estrogen Conjugates in Hen's Urine.
Private Communications.

A Note on Diurnal Variation of Six-Hour Urinary Estrone Excretion by Laying Hen.

Chromatographic Identification of Estriol and 16,17-Epiestriol as Constituents of the Laying Hen.

Mathur, R.S. and Common, R.H., 1968a.
A Note on the Daily Urinary Excretion of Estradiol-17β and Estrone by the Hen.
Mathur, R.S. and Common, R.H., 1968b.
Metabolism of Steroid Estrogens in the Hen. Conversion In Vivo of Estradiol-17β-4-14C-17α-3H to 16-Epiestriol-4-14C-17α-3H. Steroids (In Press).

Urinary Excretion of Estrone and of 16-epi-Estriol plus 17-epi-Estriol by the Hen.

Mayer, C., 1952.
Recherches Experimentales sur les Role du Foie dans le Metabolisme des Oestrogènes.

Quantitative Estimation and Identification of Estrogens in Bovine Urine.

Some Aspects of the Metabolism of 16-C14-Estrone in Normal Individual.

The Isolation and Estimation of Steroid Oestrogens in Placental Tissue.

Conversion of 16-c14-Estradiol-17β to C14- Labeled Estriol by Avian Liver Slices.

Enzymatische Demethylierung von 2-Methoxyestrogenen.
Naturwiss., 49: 328.
Cited from Chem. Abstr., 57: 11550c.

Steroidal Estrogens.

Mueller, G.C. and Rumney, G.
Formation of 6β-Hydroxy and 6-Keto Derivatives of Estradiol-16-C14 by Mouse Liver Microsomes.


Isoléent et Identification de Stéroides Chez les Vertébrés
Inférieurs et les Oiseaux.

Untersuchungen über den Stoffwechsel von Steroidhormonen bei
Vertebraten V.
Stoffwechsel Phenolischer Steroide in der Leber des Huhnes In Vivo.

Pangels, G. and Breuer, H.
Cellular Location of 17β-Estradiol-16α-Hydroxylase.
Naturwiss., 49: 106-107 (in German)
Cited from Chem. Abstr., 57: 2790c

Marshall's Physiology of Reproduction.

The Metabolism of α-Estradiol In Vitro.

The Isolation of Estrone from the Bile of Pregnant Cows.

Pearlman, W.H., Rakoff, A.E., Paschikis, K.E., Cantarow, A. and Walkling, A.A.
1948.
The Metabolic fate of Estrone in Bile Fistula of the Dogs.

Urinary Oestrogens in the Stallion. Qualitative and Quantitative Investigations.

Urinary Oestrogens in the Mature Male Mule.

Metabolism of Estrone in Men and Non-Pregnant Women.
Endocrinology, 31: 507-514.

The Intermediary Metabolism of the Sex Hormones.
Vitamins and Hormones, 1: 293-343.
The Biogenesis of Primary Sex Hormones.
1. The Fate of Estrins Injected into the Rabbit.

Artspezifische Unterschiede im Gehalt der Erythrocyten an
Oestradiol-Dehydrogenasen.
Naturwiss., 47: 3.
Cited from Chem. Abstr., 54: 16502d.

Hydroxysteroid- Dehydrogenasen, \( \Delta^5 \)-3-Ketosteroid-Isomerase und
\( \Delta^4 \) Steroid-Hydrogenase in Raten Blutzellen.

Prelog, V. and Fuhrer, I., 1945.
Über die Isolierung von 3-Desoxy-Equilenin aus dem Harn Trächtiger
Stuten.
Cited from Chem. Abstr., 40: 15713

Raud, H.R., 1968.
Metabolism of Natural Estrogens by Avian Liver Tissue Preparations
In Vitro.

Interconversions of 16-Epioestriol and Oestriol by Avian Liver
Tissue In Vitro.

In Vitro Biosynthesis of Steroid Sulfates by Cell-Free Preparations
from Tissue of the Laying Hen.

In Vitro Metabolism of Estrone-4-\( ^{14} \)C and Estrone -6,7-\( ^3 \)H-3-Sulfate
by Laying Hen Liver Homogenates.

The Partial Purification of 17\( \alpha \) and 17\( \beta \) Estradiol Dehydrogenase
Activities from Chicken Liver.

Richardson, G.S., Welicky, I., Batchelder, W., Griffiths, M. and Engel, L.L.,
1963.
Radioautography of \( ^{14} \)C and \( H^3 \) Labelled Steroids on Thin Layer
Chromatograms.
J. Chromatog., 12: 115-118.
Rumney, G., 1956.  
Metabolism of Estradiol-16-C$^{14}$ by Fortified Enzyme Systems.  


Ryan, K.J. and Engel, L.L., 1953.  
The Interconversion of Estrone and Estradiol-17β by Rat Liver Slices.  
Endocrinology, 52: 277-286.

Isolation of Steroids from a Feminizing Adrenal Carcinoma.  

The Estrogens of the Pregnant Mare.  
Endocrinology, 68: 411-416.

Schiller, J. and Pincus, G., 1943.  
The Fate of α-Estradiol and of Estriol Injected into a Human Male Subject.  

Separation and Estimation of Estradiol-17α.  
Endocrinology, 74: 870-877.

Schwenk, E. and Hildebrandt, F., 1932.  
Ein Neues Isomeres Follikle Hormon aus Stutenharn.  

Metabolism of Oestrone and Oestradiol in Human Foeto-Placental Unit at Mid pregnancy.  

15α-Hydroxylation: A New Pathway of Estrogen Metabolism in the Human Fetus and the Newborn.  

Urinary Metabolites of Estradiol and Estriol Administered Intraamniotically.  
Serchi, G., 1953.
The Separation of a Oestratrienic Urinary Metabolite in Crystalline Form, by Extraction from Paper Chromatograph. Chimica (Milan), 8: 9-11 (in Italian)

Metabolism of 3-Phenolic Steroid Hormones III: Ether and Water Soluble Fractions of Estrone-4-14C and its Metabolites in Chickens.
G. Biochim. 15: 105-118 (in Italian)

Chemical Method for the Determination of Estrone, Estradiol-17α and Estradiol-17β in Canine Urine.
Steroids, 1: 409-422.

Conversion of 6,73H-Estradiol-17β into Estrone and Estradiol-17α in the Mature Male Dog.

Steroids Present in the Follicular Fluid of the Mare.
J. Endocr., 20: 147-156.

Steroids Present in the Follicular Fluid of the Cow.
J. Endocr., 23: 401-411.

Steroid Concentrations in Normal Follicular Fluid and Ovarian Cyst Fluid from Cows.

Oestrogen and Progesterone Levels in the Pregnant Rhesus Monkeys.

Estrogens in Urine of Pregnant Ewes.
Endokrynol. Polska, 16: 597-607 (in Polish)
Cited from Chem. Abstr., 64: 16385.

Studies on the Phenolic Steroids in Human Subjects. 1. The Conversion of 16-C14-Estrone to C14-16-Ketoestrone


Stroud, S.W., 1939.
Recovery of Injected Oestrogenic Substances from Rabbit Urine.
J. Endocr., 1: 201-207.

'Avian Physiology'.

The Reduction In Vitro of [16-14C]-16-Keto-Estradiol-17β by Mammalian Blood.

Reduction of 16-Ketoestradiol-17β-16-14C by Mammalian Blood.

Perfusion Studies of the Human Placenta.
II Metabolism of C14-17β-Estradiol with and without Added Human Chorionic Gonadotrophin.

The Metabolism of Radioestrone in the Rat.

Reproduction in the Domestic Fowl.
Physiology of the Female.
'Reproduction in Domestic Animals'
Edited by Cole, H.H. and Cupps, P.T.

Velle, W., 1958a.
Undersøkelselser over Naturalig Forekommende Oestrogener hos Drøtyggere Og Gris.
Ph.D. Thesis, The Veterinary College of Norway, Oslo.

Velle, W., 1958b.
Further Investigations on Urinary Oestrogen Excretion by the Boar.

Velle, W., 1958c.
Isolation of Oestrone and Oestradiol-17β from the Urine of Adult Boars.
Velle, W., 1958d.  
Studies on Oestrogens in Cattle. The Metabolic Transformation of Oestradiol-17β to Oestrone and Oestradiol-17α in the Young Calf.  

Velle, W., 1958e.  
Studies on Oestrogens in Cattle. On the Metabolism of Oestradiol-17α in the Young Calf.  

Velle, W., 1959.  
Isolation of Estrone from the Urine of Pregnant Sow.  

Velle, W., 1963.  
Unpublished Observations.  

Velle, W., 1963.  
Metabolism of Estrogenic Hormones in Domestic Animals.  

Observations on the Occurrence of 16-epi Oestriol in Urine.  

Secretion of Estriol by Chick Embryo Ovaries Cultured In Vitro.  
C.R. Acad. Sci., 266: 159-160.  
Cited from Chem. Abstr., 68: 76079g.

Embryologie Chimique: Sur la Formation d'Oestrone et d'Oestradiol par les Gonades de Embryon de Poulet Femelle Cultivées In Vitro.  

Ketonic and Non-Ketonic Estrogens.  

The Isolation of Theelin from Human Placenta.  

The Ketonic Estrogen of Sow Ovaries.  
Metabolism of 17α-Estradiol-6,7-3H by Non-pregnant Women. 

Metabolism of 4-14C-17α-3H-Estradiol-17α and 16-14C-17α-3H-
Estradiol-17β by the Rabbit. 
Endocrinology, 83: 113-117.

Wintersteiner, O. and Hirschmann, H., 1937. 
Estrogenic Diols in the Urine of Pregnant Mares. 

Estrogenic Dihydroxy Compounds in the Urine of Pregnant Mares. 

Chemical Nature of δ-Follicular Hormone. 

Determination of Oestrogens in the Faeces of Sheep. 

Conversion of Dehydroepiandrosterone-7α-3H to Oestrogens by 
Corpus Luteum, Placenta and Placenta plus Foetal Viscera in Early 
Human Pregnancy. 
Acta Endocr., 54: 181-188.

Zander, J., Brendle, E.O., v Munstermann, A.M., Diczfalussy, E., 
Identification and Estimation of Oestradiol-17β and Oestrone 
in Human Ovaries. 

Zucconi, G., Lisboa, B.P., Simonitsch, E., Roth, L., Hagen, A.A. and 
Isolation of 15α-Hydroxyoestriol from Pregnancy Urine and Urine 
of the Newborn Infants. 