MEMOIRS OF THE
SOCIETY FOR ENDOCRINOLOGY

No. 10

PROGRESS IN ENDOCRINOLOGY

PART II

EDITED BY

K. FOTHERBY, J. A. LORAINE,
J. A. STRONG & P. ECKSTEIN

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PART II. BIOCHEMISTRY AND BIOLOGICAL ACTIONS OF STEROIDS AND OTHER HORMONES

Proceedings of the Edinburgh meeting on Endocrinology, 16–20 August 1959

Edited on behalf of the Society for Endocrinology by
K. FOTHERBY, J. A. LORAINE, J. A. STRONG AND P. ECKSTEIN

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FOREWORD

Memoirs 9 and 10 report the proceedings of the Edinburgh meeting on Endocrinology, held in August 1959. This was the first occasion on which the British Society for Endocrinology held a joint meeting with the Endocrine Societies representing the 'Acta Endocrinologica Congresses'. Approximately four hundred delegates from some thirteen countries attended.

The conference was most successful, both socially and scientifically, and proved an encouraging landmark in the relations between British endocrinologists and their 'Acta' colleagues.

The meeting consisted of four symposia dealing with neuroendocrinology, endocrinology of the thyroid and of the parathyroid glands and endocrinological aspects of cancer. In addition, about seventy short papers covering a wide variety of topics were read. In view of the large and diverse character of the material communicated, it was decided to publish the proceedings as two separate volumes.

Memoir 9 reports recent work on neuroendocrinology, the thyroid and parathyroid glands. Memoir 10 contains a review of advances in oestrogen biochemistry during the last decade, a discussion of the relationship of the endocrine glands to cancer, and a variety of papers dealing with the biochemistry and effects of steroid and protein hormones.

The Congress met under the presidency of Sir Charles Dodds, F.R.S., Chairman of the Society for Endocrinology. The local organization was in the hands of a Committee under the chairmanship of Professor G. F. Marrian, F.R.S., the local secretary being Dr J. A. Loraine.

The Society wishes to record its indebtedness for the help and generous support received from:

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In the preparation of these Memoirs the help of Mrs Grace Auld and Mrs May Nalodka, Conference Secretaries, and Miss Heather Paterson, Editorial Secretary, was much appreciated.

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SOME RECENT ADVANCES IN OESTROGEN BIOCHEMISTRY

By G. F. MARRIAN

Since this lecture is to be my 'swan song' as an endocrinologist in Edinburgh, I thought it might be appropriate for me to review recent progress in a field of research which has been of absorbing interest to me throughout my stay in this University and for more than 10 years before I came here. It also seemed that it might be appropriate for my review to cover the past decade: for the first year of that decade—1949—was when research on the biochemistry of the oestrogens took on a new lease of life in Edinburgh as a result of the stimulus provided by two colleagues who came to work with me from New Zealand and Canada respectively—J. B. Brown and the late W. S. Bauld.

To begin with I want to mention briefly the advances in quantitative oestrogen methodology which were made in Edinburgh during the period covered by this review:

In 1947 the Medical Research Council established a Clinical Endocrinology Research Unit in Edinburgh; and at the outset the Directors of this Unit decided that a thorough investigation of the endocrinology of the menstrual cycle should be undertaken. For this investigation we required a satisfactory quantitative method for the daily determination throughout the cycle of the urinary excretion of oestradiol-17β, oestrone and oestriol, which at that time were the only compounds of the oestrogen group known to be present in the urine of human subjects. Bioassay methods were quite clearly too time-consuming and laborious for routine daily use—and also too inaccurate; while chemical methods, based on the well-known Kober colour reaction, although of limited value for the determination of these oestrogens in the urine of pregnant women, were at that time virtually of no value for the determination of the much smaller concentrations of the oestrogens in menstrual cycle urines (Marrian, 1948). Accordingly there was only one thing to be done—we had to develop a satisfactory and convenient chemical method ourselves.

From 1947 to 1949 my co-workers and I worked on this methodological problem with a conspicuous lack of success; and then, just as I was ready to admit defeat Brown and Bauld came to Edinburgh full of eagerness to tackle this formidable problem. Less than 5 years later, in February 1954 at a symposium held in London by the Society for Endocrinology, Brown and Bauld each announced the development of methods based on the Kober reaction for the quantitative determination in urine of oestradiol-17β, oestrone and oestriol separately in concentrations as low as about 3–5 μg of each in a 24-hr sample (Bauld, 1955; Brown, 1955*a*).

At that time and subsequently these two methods met with much criticism, some of which was justifiable and some of which was not. But all this criticism was valuable since it stimulated Brown and Bauld and others who adopted their methods to investigate sources of error in them and to introduce various modifications designed to improve their specificity, sensitivity and accuracy. And now, 5 years after they were first described, both methods in their improved forms are firmly established as reliable procedures for the determination in urine of oestradiol-17β, oestrone and oestriol (Brown, 1955*b*; Bauld, 1956; Marrian, 1956; Brown, Bulbrook & Greenwood, 1957; Gallagher, Kraychy, Fishman, Brown & Marrian, 1958).

These two methods are unquestionably of great value for the study of oestrogen excretion during the menstrual cycle; but, unfortunately they are not quite good enough for the determination of oestrogens in the urine of men and of postmenopausal

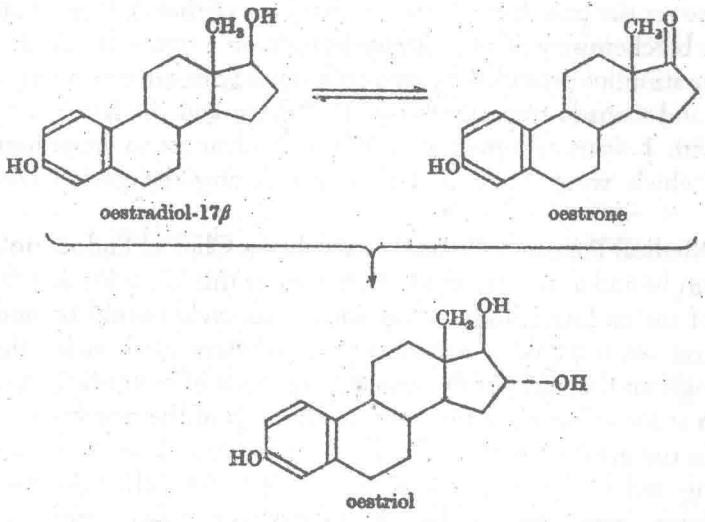

Fig. 1. Experimentally established metabolic interrelationships between oestradiol-17β, oestrone and oestriol in man (Marrian & Bauld, 1954).

women. However, as a result of further methodological advances which have been and are being made the precise and accurate determination of oestrogens in such urines may soon be possible.

Five years ago our knowledge of the quantitative aspects of the biochemistry of the oestrogenic hormone in man could have adequately been represented by the information summarized in Fig. 1. The three oestrogenic substances, oestradiol-17β, oestrone and oestriol, had been isolated from human urine (and placenta): it had been established by *in vivo* experiments on human subjects that oestradiol-17β and oestrone are readily interconvertible in the body and that oestriol is formed from one or the other of these by an irreversible process.

The quantitative aspects of these metabolic interrelationships as established by such experiments are important. It had been shown that following the administration of

either oestradiol-17β or oestrone to human subjects only about 15–20 % of the administered dose was excreted in the urine as a mixture of oestradiol-17β, oestrone and oestriol; and since there was indirect evidence to suggest that the ovarian oestrogenic hormone was oestradiol-17β or oestrone or both, it was concluded that only about 15–20 % of the hormone secreted by the ovaries during the menstrual cycle could be excreted in the urine in the form of the then-known oestrogens.

Five years ago we knew little or nothing about the fate of the 'unaccounted-for' 80–85 % of an administered dose of oestradiol-17β or oestrone. Today, however, the position is quite different. From the work of Beer & Gallagher (1955) we know that a higher proportion (65 % or more) of oestradiol-17β administered to human subjects is excreted subsequently in the urine in some form or another; and we know from the work of Levitz and his colleagues at the New York University College of Medicine, of Gallagher and his colleagues at the Sloan-Kettering Institute in New York, of my own group here in Edinburgh, and of Frandsen in Copenhagen that human urine may contain at least eleven different substances of the oestrogen group and not just three.

The discovery of these 'new' urinary oestrogen metabolites has naturally led to speculations about how they might be formed in the body from oestradiol-17β or oestrone: and these speculations have led to experiments designed to confirm or refute them. In consequence our knowledge of the metabolism of the oestrogenic hormone is now impressively greater than it was 5 years ago when all this work was started.

DISCOVERY OF THE 'NEW' URINARY OESTROGEN METABOLITES

The eight compounds of the oestrogen group which have been isolated from or detected in the urine of human subjects during the past 5 years are listed in chronological order of their discovery in Table 1.

Our own work started in 1954. A year or two earlier both Brown and Bauld, in the course of their methodological studies, had detected trace amounts of a fourth Kober chromogen additional to oestradiol-17β, oestrone and oestriol in pregnancy and menstrual cycle urines. In due course this compound was isolated and identified as 16-*epi*oestriol (Marrian & Bauld, 1954, 1955).

This isolation stimulated us to speculate about the biogenesis of oestriol and 16-*epi*oestriol; and our speculations led us to look in urine for 16-oxo-oestradiol-17β, which we thought might be the common metabolic precursor of both triols (Fig. 2). We did in fact isolate this compound, but we concluded prematurely that it was in all probability an artefact formed by the rearrangement of 16α-hydroxyoestrone which we isolated at the same time in considerably larger amounts (Marrian, Watson & Panattoni, 1957).

In the meantime 16-oxo-oestradiol-17β had been looked for and detected by reverse isotope dilution in the urine of human subjects treated with ^{14}C-labelled oestradiol-17β by Levitz, Spitzer & Twombly (1956).

Our own isolation of 16α-hydroxyoestrone led us to speculate again about the biogenesis of oestriol and 16-*epi*oestriol (Fig. 3). We suggested that 16α-hydroxyoestrone

Table 1. *Newly discovered urinary oestrogen metabolites*

16-*Epi*oestriol*	1954–5	Marrian & Bauld (1954, 1955)
16-Oxo-oestradiol-17β†	1956	Levitz, Spitzer & Twombly (1956)
16-Oxo-oestradiol-17β*	1958	Layne & Marrian (1958*a*, *b*)
16α-Hydroxyoestrone*	1957	Marrian, Watson & Panattoni (1957); Marrian, Loke, Watson & Panattoni (1957)
2-Methoxyoestrone*	1957	Kraychy & Gallagher (1957)
18-Hydroxyoestrone*	1957–9	Loke, Watson & Marrian (1957) Loke, Marrian, Johnson, Meyer & Cameron (1958) Loke, Marrian & Watson (1959)
16β-Hydroxyoestrone*	1958	Layne & Marrian (1958*a*, *b*)
16β-Hydroxyoestrone†	1958	Brown, Fishman & Gallagher (1958)
2-Methoxyoestriol†	1958	Fishman & Gallagher (1958)
2-Methoxyoestradiol-17β‡	1958	Frandsen (1959)

* Isolated.
† Detected by reverse isotope dilution after administration of ^{14}C-labelled oestradiol-17β or oestrone.
‡ Detected.

might be the intermediate between oestrone and oestriol, and we further suggested that the then-unknown compound, 16β-hydroxyoestrone, might be the intermediate between oestrone and 16-*epi*oestriol. Last year we were able to isolate 16β-hydroxyoestrone from the urine of pregnant women (Layne & Marrian, 1958*a*, *b*) at the same time as Brown, Fishman & Gallagher (1958) detected it by reverse isotope dilution in the urine of subjects treated with ^{14}C-labelled oestradiol-17β.

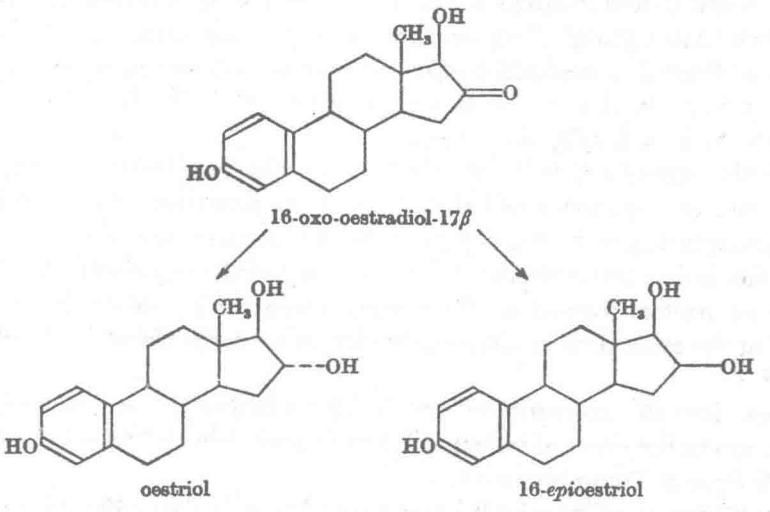

Fig. 2. Hypothetical biogenesis of oestriol and 16-*epi*oestriol by the metabolic reduction of 16-oxo-oestradiol-17β (Marrian & Bauld, 1955).

In the course of this same work (Layne & Marrian, 1958*a*, *b*) we also isolated 16-oxo-oestradiol-17β; and since the extraction process we used could not have led to the artefactual formation of this compound by rearrangement of either 16α-hydroxyoestrone or 16β-hydroxyoestrone we had to admit that the results of Levitz *et al.* (1956), which we had previously questioned (Marrian *et al.* 1957), must be valid.

The isolation of 18-hydroxyoestrone (Loke, Watson & Marrian, 1957; Loke, Marrian, Johnson, Meyer & Cameron, 1958; Loke, Marrian & Watson, 1959) was the fortunate outcome of a chance observation. In attempting to develop a quantitative

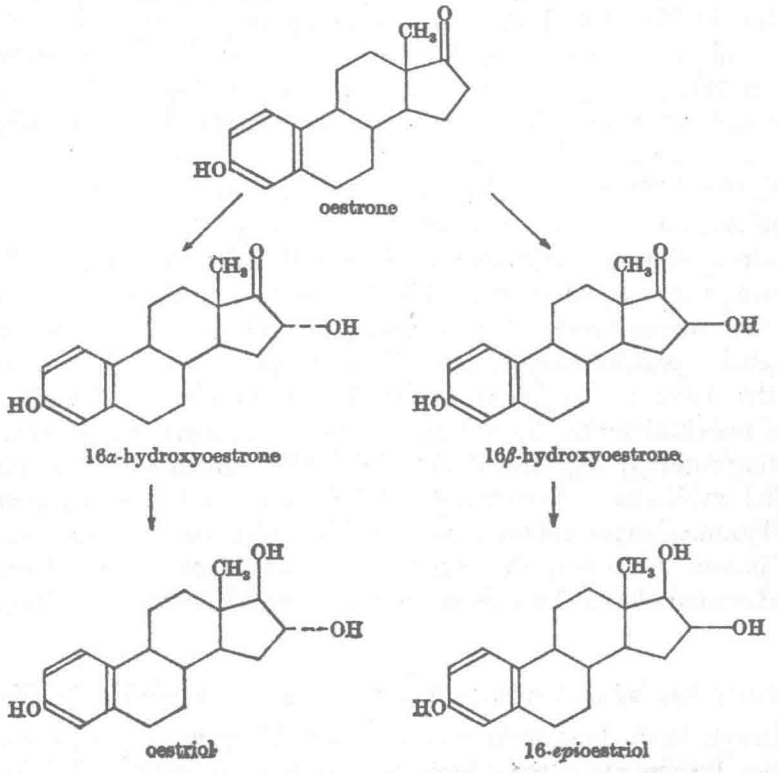

Fig. 3. Hypothetical biogenesis of oestriol and 16-*epi*oestriol from oestrone (Marrian, Watson & Panattoni, 1957; Marrian, Loke, Watson & Panattoni, 1957).

method for the determination of the ring D α-ketolic oestrogen derivatives we carried out a column partition chromatogram on a ketonic-phenolic fraction of pregnancy urine when we observed trace amounts of a Kober-chromogen which was appreciably more polar in its behaviour than either 16α-hydroxyoestrone or 16-oxo-oestradiol-17β. This was provisionally designated KC–6. However, after preparing a chromatographically purified concentrate of KC–6 from a large volume of urine we found that it contained two different Kober-chromogens which could be fairly well separated from one another by virtue of their different solubilities in cold chloroform. The more sparingly soluble of these (KC–6A) was obtained as a crystalline compound, and we

advanced evidence which indicated that it might be 18-hydroxyoestrone (Loke *et al.* 1957). Subsequently, in collaboration with Johnson and his colleagues at Wisconsin, we obtained conclusive evidence that our tentatively advanced structure was indeed correct (Loke *et al.* 1958, 1959).

Its structure suggested that 18-hydroxyoestrone might be a metabolite of oestrone formed by 18-hydroxylation in the adrenal gland since Kahnt, Neher & Wettstein (1955) have demonstrated 18-hydroxylation of 11-deoxycorticosterone on incubation with ox-adrenal homogenates. Indeed we were able to show (Loke *et al.* 1957) that on incubation of oestrone with ox-adrenal homogenates a product is formed in small yield which resembles 18-hydroxyoestrone chromatographically and in other respects.

The more chloroform-soluble of the two Kober-chromogens in KC–6 (KC–6B) has given us some difficulty, and we are far from certain that we have yet obtained it as a pure compound. We have some reasons to think, however, that it may contain one of the 6-hydroxyoestrones.

The work of Gallagher and his colleagues, which resulted in the discovery of 2-methoxyoestrone and 2-methoxyoestriol was part of a systematic attempt to identify the 'missing' urinary oestrogen metabolites following the administration of oestradiol-17β.

On carrying out a countercurrent distribution on a urine extract from a subject following the administration of ^{14}C-labelled oestradiol-17β, Kraychy & Gallagher (1957) detected a peak of radioactivity corresponding to a substance less polar in its behaviour than oestrone. In a further experiment this unknown oestradiol metabolite was isolated and characterized by orthodox methods as 2-methoxyoestrone.

This finding naturally suggested the possibility that 2-methoxyoestriol might also be an oestradiol metabolite. Accordingly 2-methoxyoestriol was synthesized by the method of Fishman (1958) and the product used in an attempt to detect the compound by reverse isotope dilution in the urine of a subject following the administration of ^{14}C-labelled oestradiol-17β. This attempt was successful (Fishman & Gallagher, 1958).

BIOGENESIS OF THE RING D-DISUBSTITUTED OESTROGEN METABOLITES IN MAN

Our speculations on the biogenesis of oestriol and 16-*epi*oestriol (Fig. 3) received some support when Brown and I (1957) showed by *in vivo* experiments on human subjects that administered 16α-hydroxyoestrone gives rise to urinary oestriol in high yield. They received further support when Layne and I (1958*a*, *b*) isolated 16β-hydroxyoestrone from pregnancy urine and when Gallagher and his group (Brown *et al.* 1958) demonstrated the formation of 16β-hydroxyoestrone from administered oestradiol-17β.

In the meantime, however, 16-oxo-oestradiol-17β had been brought back into the picture as a result of further work by Levitz and his colleagues. These workers (Levitz *et al.* 1958) administered ^{14}C-labelled oestriol to a human subject, and by reverse isotope dilution were able to detect ^{14}C-labelled 16-oxo-oestradiol-17β and 16-*epi*oestriol in the urine. From these results it is clear that oestriol can be oxidized at C–16 to yield 16-oxo-oestradiol-17β and that the latter can be reduced at C–16 to yield 16-*epi*oestriol. It is noteworthy that in these experiments Levitz *et al.* could detect no ^{14}C-labelled

16α-hydroxyoestrone in the urine. Evidently, therefore, the oxidation of a 17β-hydroxyl group, which occurs readily in the body with oestradiol-17β, is inhibited when a 16α-hydroxyl group is also present.

During the past year our ideas about the metabolic interrelationships of the urinary ring D-disubstituted oestrogen derivatives have been confirmed in part by the important *in vitro* studies of Breuer and his co-workers at Bonn (Breuer, Nocke & Knuppen, 1958*a*; Breuer, Knuppen & Nocke, 1959). The results obtained by these workers (Fig. 4) have also somewhat complicated the picture:

16α-Hydroxyoestrone was found to yield oestriol and, rather surprisingly, a small proportion of 17-*epi*oestriol. This is somewhat surprising since oestradiol-17α has so far not been detected in human urine, and since we have ourselves searched for 17-*epi*oestriol in pregnancy urine and have completely failed to find it. 16β-Hydroxyoestrone yielded 16-*epi*oestriol and 16-oxo-oestradiol-17β; and it can be assumed that

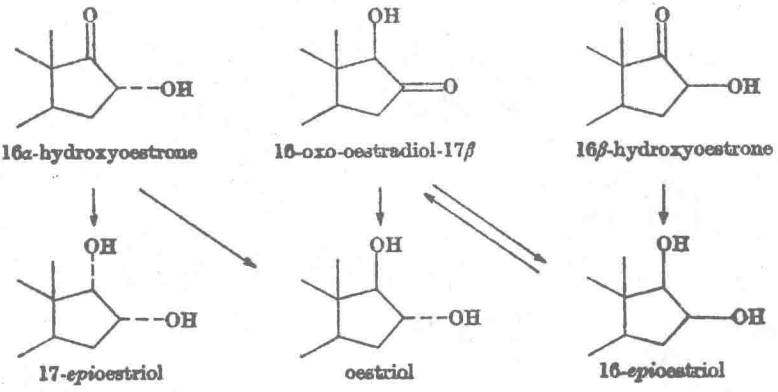

Fig. 4. *In vitro* reduction of the ring D α-ketolic oestrogen derivatives by human liver slices (Breuer, Nocke & Knuppen, 1959; Breuer, Knuppen & Nocke, 1959).

the latter was formed from 16-*epi*oestriol by oxidation at C–16. And finally 16-oxo-oestradiol-17β was found to yield a mixture of oestriol and 16-*epi*oestriol.

In all our speculations on the biogenesis of the ring D-disubstituted oestrogen metabolites we had assumed that the immediate precursor of the latter was oestrone rather than oestradiol-17β; but I must make it clear that we had no experimental basis for this assumption. Recently, however, clear-cut evidence bearing on this point has been obtained by Gallagher and his colleagues (Fishman, Bradlow & Gallagher, 1959).

These workers administered to a human subject a mixture of oestradiol-17β labelled with tritium and oestrone labelled with ^{14}C. Urine samples were then collected at frequent intervals, and, after the addition of 'carrier' amounts of pure oestrogens the oestradiol-17β, oestrone, oestriol and 16-*epi*oestriol fractions were separated from the successive samples and crystallized to constant radiochemical purity. It was found that the ^{14}C/tritium ratio for successive samples of the isolated oestrone approached that of the administered mixture more rapidly than did that of successive samples of the isolated oestradiol-17β. Furthermore, in the early stages of the experiment when the