# ON THE NATURE AND METABOLISM OF OESTROGENS IN THE DOMESTIC FOWL

A Thesis

by

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# 1. GENERAL INTRODUCTION

It is recognised that oestrogenic action plays a central role in the physiology and biochemistry of avian reproduction. The hypertrophy and functions of the oviduct, the production of yolk material by the liver, and the involvement of the bony skeleton in the metabolism and transport of the materials of the egg yolk and egg shell are among the more obvious of the functions involved. These matters have been the subject of reviews by Lorenz (1954), Sturkie (1958), Urist (1959) and van Tienhoven (1959). They have also been the subject of various studies in this laboratory in the course of the past twelve years. There are numerous indications that many more subtle, and unexplored relationships exist.

It has become increasingly evident that effective studies in this field call for information about the nature and amounts of the oestrogens excreted by the fowl, and of their metabolic inter-relationships. These matters have been the subject of investigations in this laboratory by Layne (1957) who succeeded in identifying progesterone in extracts of ovarian tissue from laying hens. He was also able to detect the presence of oestradiol, oestrone and oestriol in extracts of ovaries from laying hens. Work in this field was continued by MacRae (1960), who succeeded in

the identification of oestrone, oestradiol, and oestriol in avian droppings and the isolation of crystalline oestradiol from the same source. MacRae (1960) also carried out experiments on the metabolism of oestrogens by the laying hen by the use of $C^{14}$ labelled oestradiol. He showed that the laying hen can convert oestradiol into oestrone, oestriol and 16-epioestriol; and the administration of radioactive oestriol led to the excretion of radioactive 16-oxooestradiol-17β, radioactive 16-epioestriol and radioactive oestriol in the urine. No other radioactive metabolite could be detected. In the same experiment, the faeces from the hen yielded radioactive oestriol, radioactive 16-epioestriol, very small amounts of radioactive 16-oxooestradiol-17β and another radioactive metabolite, which was not identified, but which was not oestradiol-17β. It was found that a large proportion of the radioactive metabolites of injected radioactive oestriol was excreted by the faecal route. Equol, which is believed to represent a metabolite of plant isoflavones, was also identified in the oestrogen extracts from urine and faeces.

Frequent reference to these results is made in this thesis, which is concerned, first, with extension of knowledge as to the kinds of oestrogen excreted by the fowl; and, second, with a qualitative and quantitative study of the overall metabolism of oestrone-16-$C^{14}$ in the fowl *in vivo*.

# 2. HISTORICAL REVIEW

## 2. 1. Isolation of Oestrogens.

The chemistry of the gonadal hormones began as part of the dramatic expansion of steroid chemistry, that took place some thirty years ago. In 1929, two groups working independently, reported the isolation of a crystalline steroid oestrogen from the urine of pregnant women (Doisy, Veler & Thayer, 1929; Butenandt, 1929). This substance is now known as oestrone. In the following year Marrian (1930a, 1930b) and Doisy et al.(1930) reported the isolation of oestriol from the same source.

At this time work was in progress in England and in Germany on the isolation and structure of Vitamin D (calciferol and ergocalciferol). X-ray chrystalographic studies by Bernal (1932a, 1932b) on ergosterol and its irradiation products prompted Rosenheim & King (1932a, 1932b) to propose the perhydrocyclopentanophenanthrene formula for cholesterol. This new theoretical structure had an immediate and clarifying effect on the chemistry of a multitude of compounds, including the gonadal hormones. It was not surprising, therefore, that Marrian & Haslewood (1932a) and Butenandt (1932) in 1932 put forward structural formulae for oestrone and oestriol, that have been proved to be correct.

A suspicion arose that oestrone might not be the

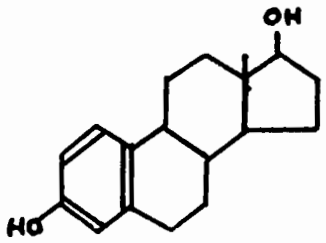
primary hormone when it was shown that oestradiol-17β, prepared by the reduction of oestrone, was physiologically more active than oestrone. This observation led to the isolation of oestradiol-17β by MacCorquodale, Thayer & Doisy (1935, 1936). They succeeded in isolating some 12 mg. of oestradiol-17β from four tons of sows' ovaries, a yield that accounted for about half of the oestrogenic activity present. Smith et al, (1939) & Huffman et al.(1940) succeeded subsequently in isolating oestradiol-17β from the urine of pregnant women.

No further phenolic steroids were isolated from human pregnancy urine between 1940 and 1955, although much had been accomplished in the laboratories of Huffman & Gallagher with regard to the synthesis of the oestrogenic steroids isolated from human pregnancy urine subsequent to 1955.

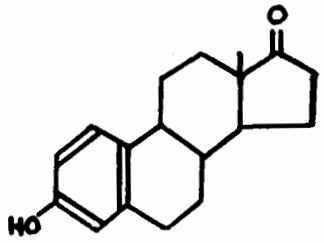
The discovery of the new oestrogens in human urine has been facilitated by a number of factors. Many, but not all, of them give a pink colour in the Kober reaction. This colour reaction is highly specific for the natural oestrogens and, in fact, was of major importance analytically in the discovery of the new oestrogens isolated by Marrian's group in Edinburgh. Isotopic labelling of oestrogens has provided a means of identification of their metabolic products.

Oestrogens isolated as natural products from human pregnancy urine

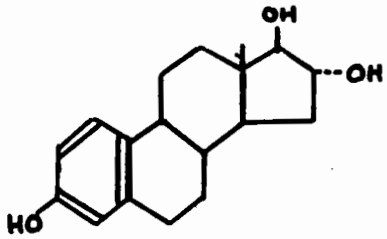
THE 'CLASSICAL' OESTROGENS



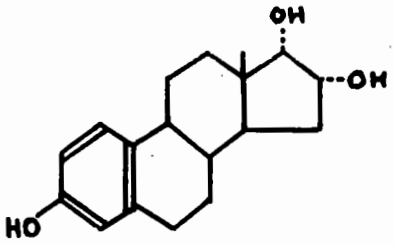

Oestradiol-17$\beta$



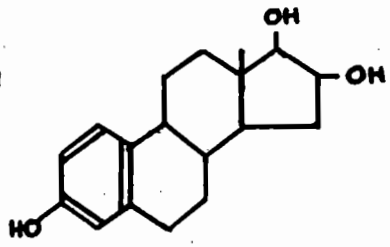

Oestrone



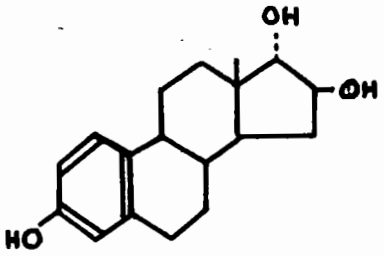

Oestriol




17-epioestriol

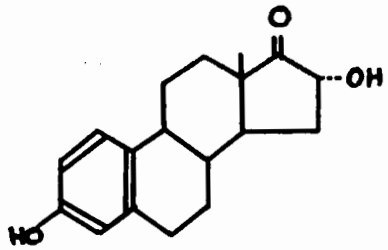



16-epioestriol

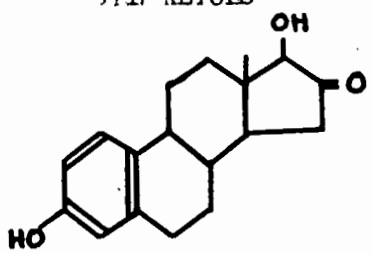



16,17, epioestriol

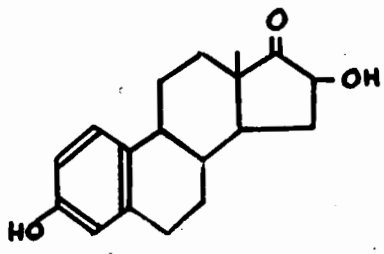
THE KETOLS



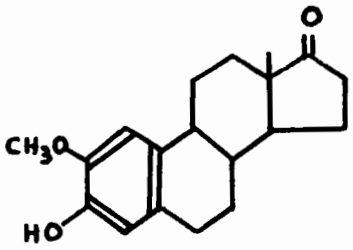

16$\alpha$-hydroxyoestrone



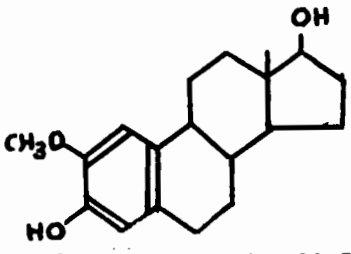

16-oxooestradiol-17$\beta$



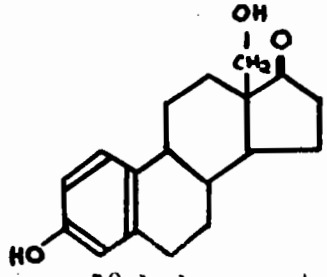

16$\beta$-hydroxyoestrone




2-methoxyoestrone



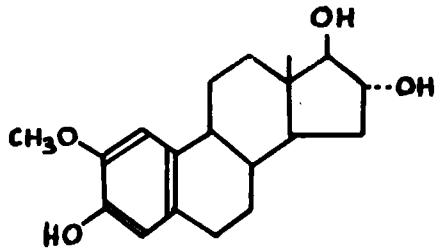

2-methoxyoestradiol-17$\beta$



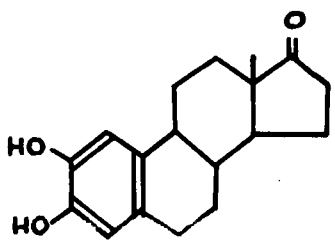

18-hydroxyoestrone

Figure 1. Oestrogens present in human pregnancy urine

Oestrogens isolated as products of injection of oestradiol-17$\beta$ -16-$C^{14}$




2-methoxyoestriol




2-hydroxyoestrone

Figure 1. continued. Oestrogens present in human pregnancy urine

16-epioestriol. (Fig. 1)

The search for hitherto unrecognized compounds of the oestrogen group in human urine had been started some time before the publication of the findings of Beer & Gallagher (1955a, 1955b). Marrian & Bauld (1954, 1955) isolated a fourth Kober chromogen which had previously been detected chromatographically during the separation of oestradiol, oestrone and oestriol. They showed that this chromogen was identical with 16-epioestriol, a compound which had previously been obtained synthetically from oestrone by Huffman and his co-workers (Huffman, 1942; Huffman & Lott, 1948).

The Ring D α-ketolic Oestrogens.(Fig. 1)

Marrian & Bauld (1955) suggested that urinary oestriol and 16-epioestriol might both arise from the metabolic reduction of 16-oxooestradiol-17β. The latter derivative was not known at that time to occur naturally but it had been prepared synthetically by Huffman & Lott (1948). Huffman & Grollman (1947) had suggested that 16-oxooestradiol-17β might be an intermediate in the metabolic formation of oestriol from oestrone. Following up these hypotheses, Watson & Marrian (1955) began a search for 16-oxooestradiol-17β in pregnancy urine and were able to detect in urinary extracts a ketonic Kober chromogen which could not be separated from 16-oxooestradiol-17β by partition chromatography in the particular solvent systems which were used. This Kober

chromogen was later isolated by Marrian et al. (1957a, 1957b) and was shown to be identical with 16α-hydroxyoestrone, a compound which had been synthesised previously by Leeds, Fukushima & Gallagher (1954). Since it was found that 16α-hydroxyoestrone in alkaline solution undergoes fairly rapid rearrangement into 16-oxooestradiol-17β, Marrian's group concluded that the 16-oxooestradiol-17β found in their earlier work (Watson & Marrian, 1955) had arisen by isomerization from 16α-hydroxyoestrone during exposure to alkali.

In the meantime Levitz et al. (1956) reported the detection, by reverse isotope dilution, of radioactive 16-oxooestradiol-17β in the urine of human subjects after the administration of oestradiol-17β-16-$C^{14}$; and it is important to note that no treatment with alkali was involved in the fractionation process. Marrian et al. (1957a, 1957b) questioned this claim in view of their conclusion that pregnancy urine contained no 16-oxooestradiol-17β other than that which was formed artefactually. This criticism of the work of Levitz et al. (1956) was unreservedly withdrawn when Layne & Marrian (1958) succeeded in isolating 16-oxooestradiol-17β as well as 16α-hydroxyoestrone from pregnancy urine extracts, by means of a procedure which, as far as they could determine, could not lead to the artifactual formation of the former compound.

Following their isolation of 16α-hydroxyoestrone, Marrian et al.(1957a, 1957b) suggested that this compound, formed by 16α-hydroxylation of oestrone, might be the metabolic precursor of oestriol and, similarly, that 16β-hydroxyoestrone might be the metabolic precursor of 16-epioestriol. At that time, this suggestion was entirely speculative but was considered sufficiently plausible to warrant a search for 16β-hydroxyoestrone in human pregnancy urine. In consequence of this search, Layne & Marrian (1958) were able to report the isolation, in crystalline form, of 16β-hydroxyoestrone from human pregnancy urine. At the same time, Brown, Fishman & Gallagher (1958) detected radioactive 16β-hydroxyoestrone by reverse isotope dilution in the urine of a subject to whom oestradiol-17β-16-$C^{14}$ had been administered.

It may be useful at this point to point out that the three ring D α-ketolic oestrogen derivatives present in the urine are unstable compounds, and that special methods have to be employed for their detection and isolation. They are largely destroyed in the course of acidic hydrolysis of urine; and, accordingly, enzymic hydrolysis of the urine has to be employed prior to their extraction with ether. Furthermore, separation into ketonic and non-ketonic fractions by the Girard reaction with trimethylammonium acetohydrazide chloride, in ethanolic acetic acid, must be carried out at room temperature, since thay are largely destroyed at elevated

temperatures. 16β-Hydroxyoestrone isomerizes very rapidly in alkaline solution (N NaOH) to 16-oxooestradiol-17β; 16α-hydroxyoestrone undergoes the same isomerization less rapidly; and 16-oxooestradiol-17β itself undergoes slow autoxidation to marrianolic acid.

16-oxooestrone (Fig.1)

Serchi (1953) reported the identification of 16-oxooestrone in pregnancy urine. Later, Slaunwhite & Sandberg (1956) reported the detection of 16-oxooestrone-16-$C^{14}$ from urine after administration of oestrone-16-$C^{14}$ to a young woman in the luteal phase of the menstrual cycle. However, they were not able to detect the compound in the urines of two similarly treated women who were not in the luteal phase.

17-epioestriol & 16, 17-epioestriol (Fig. 1)

In the course of their *in vitro* studies on the metabolism of oestrogens, Breuer and his co-workers showed that 16α-hydroxyoestrone could be reduced to 17-epioestriol by human liver slices (Breuer, Nocke & Knuppen, 1958a). In another study Breuer & Nocke (1959) showed that the fourth possible isomer of oestriol, 16, 17-epioestriol, was formed preferentially from 16-oxooestradiol-17α when the latter compound was incubated with normal human liver slices.

In view of these findings, Breuer and his co-workers attempted to isolate these two isomeric triols from human pregnancy urine. In the latter part of 1959, Breuer & Pangels

(1959) reported the isolation of 16, 17-epioestriol from human pregnancy urine in amounts equivalent to 40-60 micrograms per litre, as estimated by the Kober reaction. Later, Breuer (1960) succeeded in isolating from the same source 17-epi-oestriol in amounts corresponding to 10 micrograms per litre, as estimated by the Kober reaction.

Other newly discovered oestrogen metabolites in urine.

18-hydroxyoestrone (Fig. 1)

The isolation of an oestrogen derivative from human pregnancy urine of a rather unexpected type was reported by Loke, Watson & Marrian (1957). By partition chromatography a ketonic Kober chromogen (KC-6A) was detected in pregnancy urine which was more 'polar' than either 16α-hydroxyoestrone or 16-oxooestradiol-17β. This Kober chromogen was isolated in crystalline form and evidence was presented which indicated that it was 18-hydroxyoestrone. Proof of the correctness of the structure was obtained by Loke et al.(1958). 18-Hydroxy-oestrone, when treated with alkali at room temperature, yielded a solid product which was shown to be identical with one of the two 18-noroestrones isomeric at C-13 which were prepared synthetically. (Loke et al.1957, 1959).

It seemed possible that the 18-hydroxyoestrone may be an oestrogen metabolite formed in the adrenal gland; and, indeed Loke, Watson & Marrian (1957) showed that a compound

indistinguishable from 18-hydroxyoestrone was formed when oestrone was incubated with ox adrenal homogenates.

Concentrates of 18-hydroxyoestrone prepared from human pregnancy urine by Loke, Watson & Marrian(1957) were shown to contain a second Kober chromogen (KC-6B) which could be separated from 18-hydroxyoestrone by reason of its greater solubility in cold chloroform. This Kober chromogen has been obtained in crystalline form and it is probable that it is a 6-hydroxyoestrone (Marrian 1958, Marrian & Sneddon 1960), although it has not yet been fully characterised.

2-methoxyoestrogens & 2-hydroxyoestrogens (Fig.1).

In 1957 Kraychy & Gallagher (1957a, 1957b) announced the isolation from urine of another oestrogen metabolite of unexpected type. When extracts of urine from subjects who had been treated with oestradiol-17β-16-$C^{14}$ were fractionated by counter current distribution, a zone of radioactivity was detected which indicated the presence of an oestrogen metabolite less 'polar' than oestrone. This metabolite was isolated and identified as 2-methoxyoestrone (Fig.1). This finding was confirmed by Engel, Baggett & Carter (1957). More recently Loke & Marrian (1958) isolated 2-methoxyoestrone from human pregnancy urine. Fishman & Gallagher (1958) reported the formation in vivo of 2-methoxyoestriol (Fig.1) in human subjects following the injection of oestradiol-16-$C^{14}$ and more recently Frandsen (1959) has succeeded in

isolating 2-methoxyoestrone and 2-methoxyoestradiol (Fig. 1) from human pregnancy urine. In 1960, King (1960) showed that when oestriol is incubated with rat liver preparations in the presence of oxygen, two phenolic metabolites could be obtained. These were identified as 2-hydroxyoestriol and 2-methoxyoestriol, by comparison of their infra red and ultra violet spectra with reference compounds. He suggested that 2-hydroxyoestriol might be an intermediary in the formation of 2-methoxyoestriol from oestriol. Furthermore, Axelrod, Narasimha and Goldzieher (1960) have demonstrated that 2-methoxyoestrone can be isolated from pooled urinary extracts, when 2-hydroxyoestradiol-17β is infused intravenously in normal post-menopausal women. They state that the process involved is a true methylation. This concept received further support when Breuer & Knuppen (1960b) showed that human liver slices could bring about the conversion of 2-hydroxyoestradiol-17β to 2-methoxyoestradiol-17β, when the former was incubated with human liver slices in the presence of S-adenosyl methionine. These suggestions have received support from the circumstance that Fishman, Cox & Gallagher (1960) have recently identified 2-hydroxyoestrone (Fig. 1) as a metabolite of oestradiol-17β in man. 2-Hydroxyoestrone is the first catecholic oestrogen to be identified as a metabolite in the human or, indeed, in any species. The amounts present are of even greater importance, since, when combined with 2 methoxyoestrone (7.2% of the neutral phenolic fraction in the subject studied)

hydroxylation in the 2 position assumes a quantitative importance which equals or even surpasses 16-hydroxylation as a metabolic pathway in the metabolism of the oestrogens in some subjects. Coombs (1960) has recently presented some data on the synthesis and chemical characteristics of these 2-hydroxy compounds.

6-hydroxyoestrogens.

In 1957 Mueller & Rumney (1957) observed that mouse liver microsomes incubated under aerobic conditions in the presence of reduced T.P.N. would hydroxylate oestradiol-17β primarily in the 6 position to form 6β-hydroxyoestradiol-17β, which in turn was converted into 6-oxooestradiol-17β and 6β-hydroxyoestrone. They speculated that this family of compounds appeared to constitute a major metabolic pathway for oestradiol-17β in mouse liver preparations.

Further interest in these compounds has been stimulated by Marrian (1958) and by Marrian & Sneddon (1960), who have suggested that the second Kober chromogen (KC-6B) isolated by Loke, Watson & Marrian (1957) during the isolation of 18-hydroxyoestrone from human pregnancy urine appeared to be identical with a 6-hydroxyoestrone.

In vitro studies by Breuer, Nocke, & Knuppen (1958b, 1959a) have shown that 6-hydroxylation of oestradiol-17β or oestrone can take place on incubation of these compounds with rat

liver slices. Furthermore, Breuer & Knuppen (1960a) have succeeded in isolating a 6-hydroxyoestradiol-17β and oestriol after incubation of oestradiol-17β in the same system. Further studies by Breuer et al. (1960) have indicated that biosynthesis of 6-hydroxylated oestrogens can originate by two metabolic pathways, viz., one which involves the conversion of 6-hydroxy C-19 steroids to corresponding C-18 steroids and a second which involves the direct hydroxylation of oestradiol-17β. Using Ryan's (1959a) enzyme preparation of human placenta, it was shown that 6α-and 6β-hydroxy $\Delta^4$-androstene-3,17-dione on incubation with human placentae could be converted into 6α-hydroxyoestrone and 6β-hydroxyoestrone respectively. In order to investigate the second metabolic pathway, oestradiol-17β was incubated with human foetal liver slices. 6'α'-Hydroxyoestradiol-17β was subsequently identified after extraction of the incubation mixture with ether-chloroform (3:1 $^{v}/_{v}$) and chromatography in the chloroform/formamide system.

It should be pointed out that the 6β configuration assigned to the hydroxyl group in the 6-hydroxyoestradiol-17β and the 6-hydroxyoestrone isolated by Mueller & Rumney (1957) may be wrong. Wintersteiner & Moore (1959) have given reasons for believing that the 6-hydroxyl group in the 6-hydroxyoestradiol which is formed by metal hydride reduction of 6-oxooestradiol-17β may have a quasi-equatorial

configuration. Accordingly, these authors suggested that the reference compound supplied to Mueller & Rumney should be tentatively described as 6α-hydroxyoestradiol-17β. Marrian and Sneddon (1960) have recently synthesised 6-oxooestriol and 6α-hydroxyoestriol and their acetates from oestriol.

Whilst all the isolations or identifications of oestrogen metabolites referred to above have been from human sources, the occurrence of oestrogens in other species has been reported. Since the literature on this aspect of the oestrogens has been reviewed by Dorfman (1957), a detailed report is not included here.

El-Attar & Turner (1957) published an extensive report on the determination of oestrogens by spectrofluorimetric methods in the urine and faeces of cows at different stages of pregnancy. They found that the non-ketonic fraction of the phenolic steroid extract was the major component in both urine and faeces, and their oestrogen was excreted mainly by way of the faeces. The total amount of oestrogen excreted in the cow was low as compared to the amount excreted in urine of the mare or to that of the human female. This finding, that oestrogen is excreted mainly *via* the faeces in the pregnant cow, has been reported by others (Pearlman *et al.* 1947). Pope McNaughton & Jones (1957) succeeded in isolating oestrone from the urine of cows in late pregnancy in amounts equivalent to 0.3 mgm per litre. Klyne & Wright (1956) also isolated

oestradiol-17α as well as oestrone from the urine of the pregnant cow, but were unable to detect oestradiol-17β either in the urine of the cow or of the goat (Klyne & Wright 1957). In the latter study they succeeded in isolating oestrone and oestradiol-17α from the urine of the pregnant goat. However, Gorski & Erb (1959) have reported the presence of both oestradiol-17β and oestradiol-17α in extracts of bovine placenta.

The urine of pregnant mares has provided a number of interesting phenolic steroids. In addition to the well known ketonic steroids, viz. oestrone, equilin and equilenin which differ only in the degree of saturation in ring B, the corresponding diols in which the hydroxyl group at C-17 has α-configuration, i.e. oestradiol-17α, dihydroequilin-17α, and dihydroequilenin-17α are also present (Hirschmann & Wintersteiner (1937), Glen et al. (1956), Gaudry & Glen, 1958).

It would seem certain, therefore, that between the pregnant mare on the one hand and man on the other there is a qualitative species difference in the way oestrone is metabolically reduced. It should be noted that the ring B unsaturated steroids have not been recognized as normal excretory products in species other than the horse, but convincing evidence has been obtained by Salhanick & Berliner (1957) for the presence of equilin in the extract from a feminizing adrenal carcinoma in man. The peculiar steroidal

ketone $\Delta^{5.7.9}$- oestra trienol-3-one-17 has been found in relatively large quantities by Glen et al.(1958) in pregnant mares' urine. The only other isolation of this compound recorded in the literature is by Heard & Hoffman (1940). Short (1960) has recently isolated oestradiol-17β and oestrone from the follicular fluid of the mare.

More recently, research groups have begun studies on animals other than the mare and bovine.

Velle (1958c)succeeded in isolating oestrone and oestradiol-17β from the urine of the adult boar, and more recently Krèstoffersen & Velle (1960) have made quantitative studies of the excretion of oestrogens in the dog and bitch. They have shown, by means of the quantitative technique of Brown (1955), that oestrogens are absent from the urine of the dog. In the case of the bitch they detected oestriol in amounts equivalent to 2.2 micrograms per litre of urine, after enzymatic hydrolysis of pooled urine from three bitches in late pregnancy, and values for oestrone and oestriol of 4.0 micrograms per litre of urine and 13.0 micrograms per litre of urine respectively, after acidic hydrolysis of fresh urine from a single bitch. Oestradiol-17β was not detected in the urine, in either case.

As regards avian species, Hurst, Kuksis & Bendell (1957) reported the presence of oestrone and oestriol in the droppings of the male and female domestic fowl. In addition,

they observed the presence of oestradiol-17β in very small amounts in the droppings of the rooster. Their identifications were based on chromatography and colour tests.

Layne, Common, Maw & Fraps (1958) identified oestrone and oestradiol-17β in the ovaries of laying hens. Their identifications were based on chromatography, ultraviolet spectrophotometry and the chromatography of the unknowns with radioactive reference compounds. In addition, they identified oestriol by chromatography and ultraviolet spectrophotometry.

More recently, MacRae, Layne & Common (1958) have identified radioactive oestrone, oestriol and oestradiol-17β in extracts of bile and droppings of the laying hen, after intravenous injection of oestradiol-17β-16-$C^{14}$. Radioautograms of the chromatograms showed the presence of oestradiol-17β in the blood but not that of any other oestrogen metabolite. In a similar study, MacRae & Common (1960) demonstrated the presence of 16-epioestriol in the droppings of a laying hen, as well as oestriol, oestrone and oestradiol-17β. The former metabolite had been detected in the first study but had not been characterized as such. Identifications in both cases were based on chromatographic evidence and the chromatography of the unknown radioactive zones with the appropriate reference steroids. MacRae, Zaharia & Common (1959) have also reported the isolation of a small amount of crystalline oestradiol-17β from the droppings of a laying hen. The isolate was

characterized by comparison of its melting point and infra red absorption curve with those of reference oestradiol-17β.

The occurrence of oestriol in avian species is of some interest in view of the fact that it has been isolated only from human sources and was, for a time, regarded as being characteristic of the human species (Merril, 1958). Dorfman (1957), however, discusses the reported identification of oestriol in species other than man and states that, "these studies remove the myth that oestriol is an exclusive product of man".

## 2. 2. Biogenesis of the Ring D Disubstituted Oestrogen Derivatives.

Heard et al.(1956) & Callow (1956) have reviewed the metabolic interrelationships of the oestrogens. Among other conversions, the sequence oestradiol-17β⇋oestrone→oestriol has been well established in a number of mammalian species. However, the isolation of 16-epioestriol by Marrian & Bauld (1955) indicated the possibility of both 16α and 16β hydroxylations in the metabolism of the oestrogens. The scheme suggested by the Edinburgh group (Marrian & Bauld, 1955, Marrian, Watson & Panattoni, 1957b Marrian, Loke, Watson & Panattoni, 1957c), as described previously, was entirely speculative in the first instance. At that time the hypothetical intermediate between oestrone and 16-epioestriol had not been

shown to occur naturally, while none of the suggested metabolic reactions had been confirmed by direct experimentation. However, these speculations now look less speculative than they did originally, for Brown, Fishman & Gallagher (1958) have shown that 16β-hydroxyoestrone is a metabolic product of oestradiol-17β; and Brown & Marrian (1957) have shown that when 16α-hydroxyoestrone is administered to human subjects about 40% of the administered dose is excreted in the urine as oestriol. This same reaction has been observed *in vitro* with rat liver slices by King & Marrian (1958).

A defect of the foregoing scheme of biogenesis was that it provides no place for 16-oxooestradiol-17β, but the position of this metabolite has been clarified by the work of Levitz, Spitzer & Twombly (1958). These workers administered oestriol-16-$C^{14}$ to human subjects and subsequently clearly demonstrated the presence of significantly high radioactivity in the 16-oxooestradiol-17β and the 16-*epi*oestriol excreted in the urine. From these results they concluded that 16-oxo-oesradiol-17β is formed by oxidation of oestriol at C-16 and that the former gives rise to 16-*epi*oestriol by reduction of the 16-oxo group. There was no evidence that oestriol could undergo oxidation at C-17 to form 16α-hydroxyoestrone. In a more recent study, Levitz, Rosen & Twombly (1960) have clarified the position of 16-oxooestradiol in the metabolic scheme still further, by showing that when 16-oxooestradiol-16-$C^{14}$

was administered to human subjects, virtually all the radioactivity excreted in the urine was present in 16-oxooestradiol-17β, oestriol and 16-epioestriol. From this evidence it may be concluded that, in conversions which are reversible, 16-oxooestradiol-17β is reduced *in vivo* to oestriol and 16-epioestriol. Levitz, Rosen and Twombly (1960) also stated that 16-epioestriol is partially converted *in vivo* to 16-oxooestradiol-17β, although this observation was not published in the paper incorporating the foregoing results.

In dealing with the metabolism of the oestrogens reference must be made to the *in vitro* work of Breuer and his co-workers. Breuer, Nocke & Knuppen (1958a) showed that when 16α-hydroxyoestrone and 16-oxooestradiol-17β were incubated with human liver slices, the former was reduced to oestriol and 17-epioestriol while the latter yielded oestriol and 16-epioestriol. Quantitative examination revealed that the 17-keto group was preferentially reduced to a 17β-hydroxyl group, while the 16-keto group formed the 16α-hydroxyl group under these conditions. Further work by Breuer, Knuppen & Nocke (1959) showed that 16β-hydroxyoestrone, when incubated with human liver slices, gave rise to 16-epioestriol, 16-oxooestradiol-17β and an unidentified metabolite. 16-Oxooestradiol-17β could not have been formed artifactually under these conditions, and thus, this points to the possibility that 16-epioestriol may have undergone partial oxidation with the formation of 16-oxooestradiol-17β. This latter fact has also

been observed by Levitz, Rosen & Twombly (1960).

Using rat or guinea pig liver slices, Breuer, Nocke & Knuppen (1959b) showed that 16-oxooestradiol-17β could in fact give rise to both oestriol and 16-epioestriol, when incubated with these slices.

A study of the metabolism of 16-oxooestrone on incubation with human liver slices by Breuer & Knuppen (1958) and Breuer, Knuppen & Pangels (1959) yielded six of the eight possible reduction products, viz., 16-oxooestrone, 16α-hydroxyoestrone, 16β-hydroxyoestrone, 16-oxooestradiol-17β, oestriol, 16-epioestriol and 17-epioestriol. Breuer concludes from this data that 16-oxooestrone is reduced to the hydroxyoestrones to about the same extent, with preferential formation of 16-oxooestradiol-17β; the further reductions of the hydroxyketones to the triols then takes place principally in the liver. These results are in agreement with thoseof Stimmel (1958), which indicated that oestriol and 16-epioestriol are metabolic products of 16-oxooestrone. Slaunwhite & Sandberg (1958) reported the presence of 16-oxooestrone as a major metabolite on injection of oestrone-16-$C^{14}$ into a woman in the luteal phase of the menstrual cycle. These results indicate that 16-oxooestrone probably lies between oestrone and the D-ketolic oestrogens. Breuer & Nocke (1959) reported the identification of oestriol-3, 16β, 17α, (16, 17-epioestriol), the fourth possible isomer of oestriol, when

16β-hydroxyoestrone and 16-oxooestradiol-17α were incubated with normal human liver slices. They observed that 16, 17-epi-oestriol was a minor metabolite of 16β-hydroxyoestrone but that 16-oxooestriol-17α gave rise preferentially to 16, 17-epioestriol. They stated, however, that the quantitative significance of this new epimer of oestriol could not be fully assessed until the position of 16-oxooestradiol-17α in the metabolic scheme is elucidated.

The use of isotopically labelled oestrogens has been applied by Gallagher and his co-workers to determine quantitatively the metabolites present in the urine and faeces after injection of a suitably labelled precursor. The results are summarized in Fig. 2.

Beer & Gallagher (1955a, 1955b) administered small doses of oestrone-16-$C^{14}$ and oestradiol-16-$C^{14}$ to human subjects and measured the rates of elimination of the radioactivity in the urine, faeces and bile. They showed that the metabolic products of oestradiol and oestrone are indistinguishable and that the two must, therefore, be rapidly interconvertible in the body. Working with patients in whom bile could be collected by means of a biliary fistula, they found that approximately 50% of the administered radioactivity was excreted in the bile within the first 12 hours and 50% in the urine. The bile normally drains into the gut and most of the biliary oestrogens are reabsorbed from there and returned to

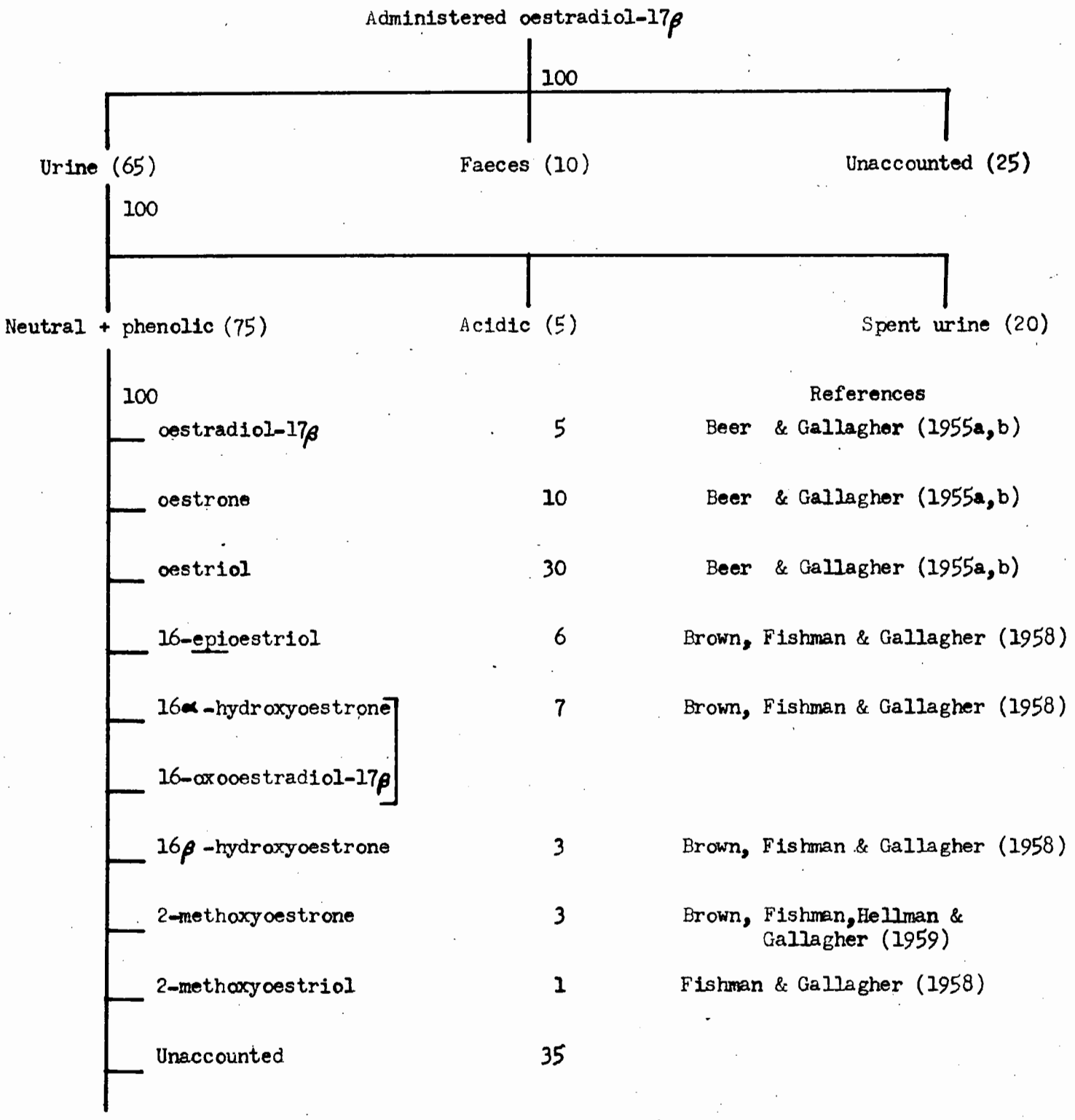



Figure 2. The excretion of oestrogen metabolites in urine (reproduced from Brown, 1959)

the liver where they are excreted again, partly in the bile and partly in the urine. Eventually, approximately 65% of the administered radioactivity was found in the urine and 10% in the faeces. Approximately 25% of the dose was unaccounted for. They showed that approximately 75% of the radioactivity present in the original urine was found in the neutral plus phenolic fractions, 5% in the acidic fraction and 20% in the spent urine. The neutral plus phenolic fraction, which contains all the known oestrogen metabolites, was further processed by countercurrent distribution and paper chromatography and separated into a number of distinct fractions. Many of these fractions were identified as the known oestrogens by their chromatographic behaviour and by reverse-isotopic dilution. The relative amounts of these oestrogens present in the neutral plus phenolic fraction, as judged by the amounts of radioactivity found in their respective fractions are shown in Fig. 2. The values have been abstracted from a number of publications by Gallagher and his co-workers. They vary considerably from subject to subject and those shown should be regarded as representative only. Thirty-five per cent of the radioactivity in the neutral plus phenolic fraction is still unidentified.

The interconvertibility of oestrone and oestradiol-17β in the body has recently been demonstrated by Fishman, Bradlow & Gallagher (1960). They administered to three patients a mixture of oestrone-16-$C^{14}$ and oestradiol-6, 7-$H^3$

in varying mass and isotope ratio. Comparison of the measured isotope ratio in oestrone and oestradiol in successive urine collections demonstrated that oxidation of oestradiol to oestrone must be more rapid than the reduction of oestrone to oestradiol. From the measured isotope ratio of other metabolites isolated it was concluded that oestriol, 16-epi-oestriol and 2-methoxyoestrone are derived from oestrone and not from oestradiol-17β.

On the basis of the evidence just presented, one may construct a scheme for the possible sequence of reactions which have actually been shown to occur, and those which are still speculative in the metabolism of the oestrogens in humans. Such a scheme is presented in Figure 3.

Both the in vivo and in vitro formation of oestriol from oestradiol-17β are well known (Dorfman, 1957). More recently, however, Ryan (1958) reported a second distinct pathway for the formation of oestriol. He demonstrated that human placental preparations are capable of converting $\Delta^5$-androstene-3β, 16α, 17β-triol to oestriol. This observation, that oestriol could be produced by aromatization of C-16 hydroxylated androgen without involving the classic conversion from oestradiol, prompted a more extensive study by Ryan (1959b) of the metabolism of C-16 oxygenated neutral and phenolic steroids by human placenta. This latter study demonstrated the conversion of three 16-oxygenated neutral steroids to

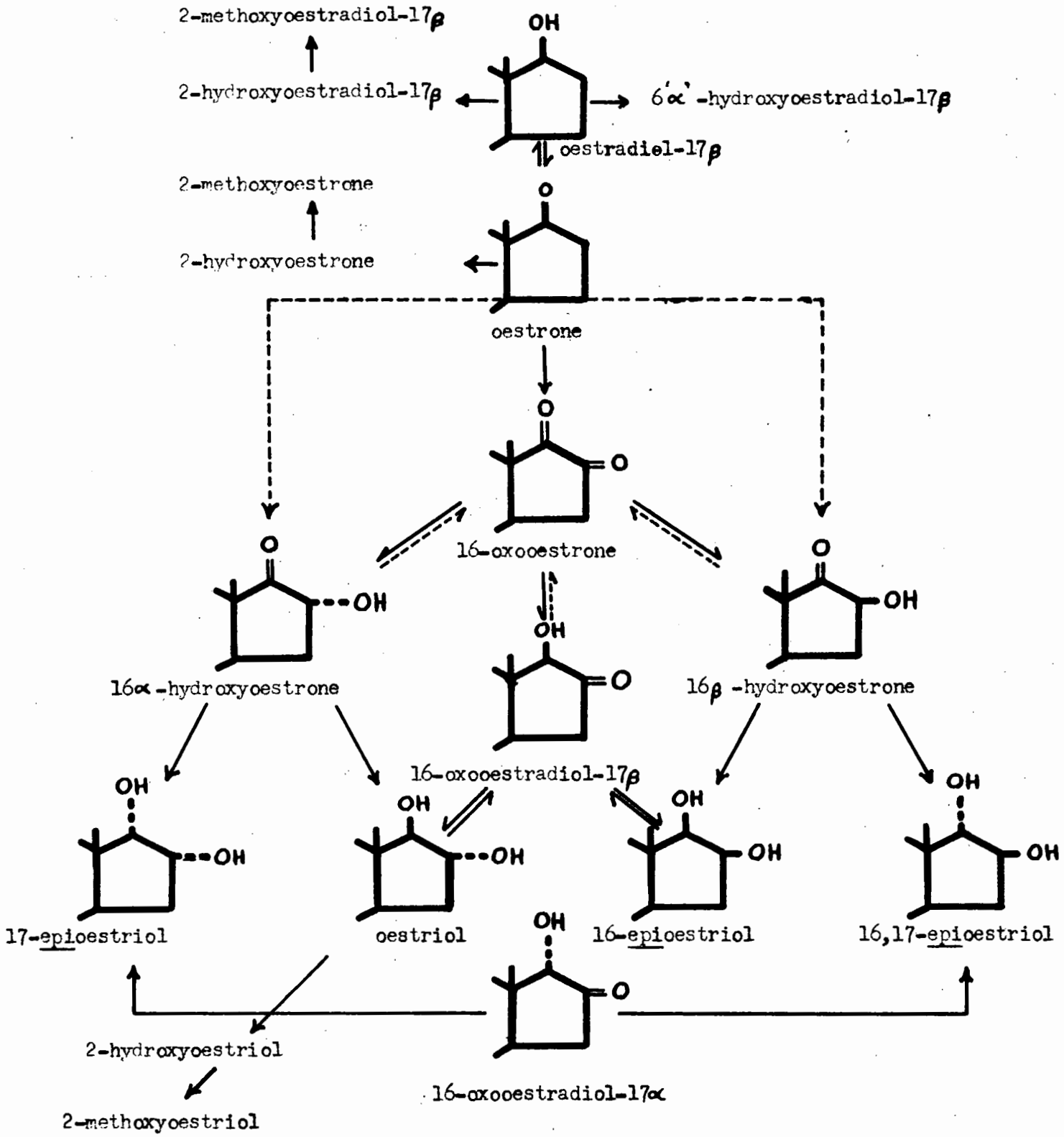



Figure 3. Schematic representation of the biogenesis of the ring A and ring D disubstituted oestrogen metabolites in man.
Solid arrows indicate reactions which have been shown to occur either in vivo or in vitro. Broken arrows indicate reactions which are still speculative.

oestriol and a new source for oestriol was established. Ryan (1959b) has also suggested a tentative intermediary role for 16α-hydroxyoestrone between the 16-oxygenated neutral steroids and oestriol. It is generally agreed that a major proportion of the oestriol in human pregnancy urine is of placental origin. Whether this fraction is produced by C-16 hydroxylation of oestradiol-17β or by the aromatization of an already 16-hydroxylated androgen must await further study. The overall conversion of androgens to oestrogens, both in vivo and in vitro, has been reviewed by Dorfman (1957) and no further discussion will be given here.

While the metabolic transformations referred to above apply to the human metabolism of oestrogens, Axelrod & Wertessen (1959, 1960) have shown that oestradiol-17β can be converted to oestrone by bovine blood and also that the principal conversion product of oestrone-16-$C^{14}$ in bovine blood is oestradiol-17α. A series of studies by Velle (1958a, 1958b, 1958d) and by Erichsen & Velle (1960) on the metabolism of oestrogens in cattle, have shown that the young calf can convert oestradiol-17β to oestrone and oestradiol-17α; oestradiol-17α to oestrone; and oestrone to oestradiol-17α. The interconversion of oestrone and oestradiol-17β was also demonstrated in vitro, in the presence of cells from amnion and endometrium and from the testicles of young bulls. Only traces of oestrone were found following incubation of oestradiol-17α in these tissues. Bovine liver cells show a poor capacity to bring about

this interconversion.

With regard to avian species, MacRae, Layne & Common (1959) provided some evidence for the validity of the metabolic sequence oestradiol⟷oestrone⟶oestriol in the laying hen by intravenous injection of oestradiol-17β-16-$C^{14}$. In a second study MacRae & Common (1960) also demonstrated the formation of 16-epioestriol on injection of the same precursor. No other metabolites of oestradiol-17β were identified in the urine since acidic hydrolysis was used to break down the oestrogen conjugates and this would have destroyed most or all of the intermediate metabolites. In a further study MacRae, Dale & Common (1960) demonstrated the *in vivo* formation of 16-epioestriol and 16-oxooestradiol-17β, but of neither oestradiol nor oestrone, after intravenous injection of oestriol-16-$C^{14}$ into the laying hen. These results are in agreement with the results and conclusions of Levitz, Spitzer & Twombly (1958) regarding the metabolism of oestriol in the human subject. The results also showed that the faeces contained appreciable amounts of radioactive 16-epioestriol and little, if any, radioactive 16-oxooestradiol-17β. The presence of the dihydroxy phenol, equol, was also demonstrated in both urine and faeces. This compound had previously been shown by Marrian & Haslewood (1932b) to be present in mares' urine, where it is believed to represent a metabolite of plant isoflavones. Mitchell & Hobkirk (1959) have demonstrated *in vitro* the conversion of oestradiol-17β-16-$C^{14}$ to radioactive oestriol by avian liver slices.

## 2. 3. METHODS OF EXTRACTION AND IDENTIFICATION OF THE OESTROGENS FROM ANIMAL SOURCES

The investigation of the steroid hormones in animal tissues or excreta involves (a) the extraction of the hormones from the particular material under study, and, (b) the purification and identification of the individual steroids or their metabolites. The amount of any individual steroid present is often minute and experimental losses are almost always considerable. It is often necessary, therefore, that the method employed should be capable of detecting and estimating amounts of the order of 5 to 100 micrograms , or even less.

The techniques used will depend on the type of material being studied and on the type of steroid or group of steroids that is being sought. Mitchell & Davies (1954) have reported an extraction procedure applicable to the isolation of oestrogen from human placenta. Bauld & Greenway (1957) reported in detail the procedures of Brown (1955),(later imporoved by Brown, Bullbrook & Greenwood 1957a, 1957b), and Bauld (1956) for the determination of oestrogens in human urine. All these methods have been adapted in the present work to the investigation of oestrogens in avian excreta.

Oestrogens, as well as other steroids, are excreted in the urine mainly in the form of water-soluble conjugates of glucuronic acid or sulphuric acid. Much of the literature on the nature and formation of these conjugates has been reviewed

by Roberts & Szego (1953) and specifically on oestrogen conjugates by Bauld & Greenway (1957). Hydrolysis of these compounds is desirable for two reasons. First, the chemical nature of all the conjugates is not yet known, so that all methods of assay have been developed for the free oestrogens. Second, extraction with organic solvents is a convenient method for the separation of oestrogens from the highly water-soluble compounds which constitute the bulk of urinary solutes. Such an extraction is facilitated by hydrolysis, since free oestrogens have much more favourable partition coefficients for extraction by organic solvents than have their conjugates. Purification is thus achieved with considerably greater convenience when the extraction is preceded by hydrolysis.

The successful recovery of steroids from their conjugates is dependent upon the establishment of conditions of hydrolysis which give optimal breakdown of the conjugates with little destruction of the steroids themselves. Bauld & Greenway (1957) have discussed the optimal conditions for hydrolysis of urinary oestrogens. The problem of destruction of oestriol, oestrone, and oestradiol-17β during hot hydrolysis has been investigated by Brown (1956). He found two indications of destruction of oestrogens when these were boiled with urine acidified with 15 volumes conc. hydrochloric acid per cent. First, the amounts of the three oestrogens released from their conjugates diminished when boiling was

continued for more than 60 minutes. Second, the recovery of free oestrogens added to urine before hydrolysis under these conditions was 10 - 20% less than when they had been added after hydrolysis. Brown (1956) also found that neither the removal of the ether-soluble material or proteins before hydrolysis, nor the addition of a variety of oxidizing or reducing agents afforded an increased yield of oestrogen from urine or changed the recovery of free oestrogens added to urine. Decrease in acid concentration and over-laying with solvent increased the recovery of added free oestrogens, but lowered the yield obtained by simple reflux of the urine specimen for 60 minutes after the addition of 15 volumes conc. hydrochloric acid per cent. On the other hand, ten-fold dilution of the urine specimen with water increased the yield of oestrogens and prevented the loss of added free oestrogens.

The advantage of enzymatic hydrolysis of urinary oestrogens over acidic hydrolysis is a lesser destruction of oestrogens. The disadvantages are the additional time required, the expense of suitable enzyme preparations, their sensitivity to inhibition and the greater tendency of enzymatic hydrolysates to form persistent emulsions in the extraction procedure. Bauld & Greenway (1957) have reviewed the various types of enzyme preparations used. Recently, Hobkirk & Cohen (1960) have compared the efficiency of four enzyme preparations containing β-glucuronidases of bacterial, mammalian and moll-

uscan origin, in liberating 17-ketosteroids in normal human urine. They showed that all four enzyme preparations were equally effective in liberating 17-ketosteroids of the 5β configuration, but that the bacterial preparation released steroids of the 5α cenfiguration more rapidly than did the molluscan preparation and with much greater ease than did the mammalian preparation. It should be noted that enzymatic hydrolysis is essential for the determination of the ring D α-ketolic oestrogens, including 16-oxooestrogens.

The purification procedures applied to extracts, are designed to separate particular groups of steroids and to remove certain contaminants. The most difficult problem in the case of urinary extracts is the elimination of pigments, which are fluorogenic or chromogenic and hence may interfere with the colour reactions employed to detect or estimate oestrogens. Cohen & Marrian (1934) introduced a procedure for the extraction, purification and separation of oestrogens from acid-hydrolysed human pregnancy urine. Their procedure wasbased on simple solvent partition. The ether extracts were washed with 10% sodium carbonate to remove the acidic fraction. This acid fraction includes various organic acids, traces of hydroxyarylcarboxylic acids and substituted hydantoins formed by condensation of urea with α-keto acids during hydrolysis. The neutral fraction includes most non-phenolic steroids, indigoids and cholesterol (Bauld & Greenway, 1957).

Bachman & Petit (1941) & Mather (1942) observed that losses of oestriol occur on washing ether with 10% sodium carbonate. Engel, Slaunwhite, Carter & Nathanson (1950) and Stevenson & Marrian (1947) suggested that sodium bicarbonate be substituted for sodium carbonate, but this gave considerably less pure fractions than the original procedure of Cohen & Marrian (1934).

Brown (1955) introduced some very effective steps in the purification of urinary oestrogen fractions. He made the important observation that the distribution of oestriol between ether and weak alkali depended more upon the ionic strength than upon the pH of the aqueous phase. Thus he found the partition coefficient of oestriol between ether and concentrated carbonate buffer of pH 10.5 to be practically the same as that between ether and saturated sodium bicarbonate solution. This permitted removal of almost as much impurity as the original wash with 10% sodium carbonate.

Bauld (1956) observed that brown pigments were formed when the ether extract from the urine was shaken with 2N sodium hydroxide following a wash with concentrated carbonate buffer. These pigments remained in the aqueous phase when the alkalinity was brought down to about pH 10 by the addition of sodium bicarbonate. The oestrogens (oestriol, oestrone and oestradiol-17β) were not decomposed by the alkaline treatment and could be re-extracted by ether from the aqueous phase at this pH.

In spite of the effectiveness of this step in purification, it was found (Bauld, 1956) that further exposure to alkali resulted in the formation of more brown pigment. Saponification in aqueous sodium hydroxide effectively removed these chromogens. Diczfalusy (1953) first demonstrated the stability of oestrone to boiling in dilute sodium hydroxide. The stability of oestrone, oestriol & oestradiol-17β to refluxing for thirty minutes in normal sodium hydroxide has since been demonstrated by Bauld (1956). The final steps in the procedure of Bauld (1956) for the determination of urinary oestrogens involve the partition of the partially purified ether extract between benzene (dissolves oestrone and oestradiol-17β) and water (dissolves oestriol). Each fraction is then submitted to partition chromatography on Celite columns.

In addition to the simple partition methods discussed above, there are several chemical methods for the purification of oestrogen fractions. All these methods involve the formation of derivatives with partition coefficients different from those of the parent oestrogens. The principle of purification by derivative formation was applied in a most efficient form by Brown (1955). After separation of oestriol, oestrone and oestradiol-17β by simple partition procedures, he formed the methyl ethers of the phenolic groups by treatment with dimethyl sulphate in alkaline solution. These derivatives were extractable from aqueous solution with

non-polar solvents and separation from residual polar contaminants was achieved in this way. Furthermore, due to the increased stability conferred on the molecules by methylation of the phenolic groups, hydrogen peroxide could be used to oxidize various impurities to polar end products.

Purification by derivative formation can also be accomplished by the use of Girard's Reagent T. This reagent was first described by Girard & Sandulesco (1936) and is used for the separation of ketonic and non-ketonic oestrogens. The ketones form water-soluble derivatives and can be separated from ether-soluble impurities by simple partition. The non-ketonic oestrogens (oestriol and oestradiol-17β) may be further purified by condensing them with succinic or phthallic anhydrides to form the corresponding hemiesters (Pincus & Pearlman, 1942). Recently Givner *et al.*(1960a) have used the Girard reaction for the quantitative fractionation and recovery of 2-methoxyoestrone, oestrone, ring D α-ketolic oestrogens, oestradiol-17β, 16*epi*-oestriol and oestriol. Excellent results were obtained by use of a modification of the method described by Pope, McNaughton & Jones (1957) and by Marrian, Watson & Panattoni (1957b). The two fractions were then subjected to partition chromatography on Celite columns.

The steroids and steroid metabolites which have been extracted and partially purified by the various procedures must then be separated and identified. In earlier work each

compound was isolated in the crystalline form and estimated gravimetrically. Chromatographic methods are now universally employed and have made possible the separation of small amounts of closely related steroids. However, in spite of the development of these newer methods crystallization is still the primary method of isolation. In some cases this may involve the processing of large amounts of material by paper chromatography before crystallization can be carried out (see Layne & Marrian, 1958).

A very useful review of the methods for separating oestrogens from hydrolysed urine is given by Bauld & Greenway (1957). The methods reviewed by them include adsorption chromatography, ion exchange chromatography, countercurrent distribution, column partition chromatography and paper partition chromatography. Another useful review of the methods of adsorption and partition chromatography of steroids and related compounds has been given by Bush (1954a, 1954b).

Filter paper partition methods are of two main types. That introduced by Zaffaroni, Burton & Keutmann(1949) uses paper impregnated with a high-boiling polar solvent, usually propylene glycol or formamide, as the stationary phase. The mobile phase is a volatile, non-polar solvent saturated with the stationary solvent. Bush (1952) used a second type of system, in which both phases consisted of volatile solvents, the stationary polar phase being preferentially adsorbed on to

the paper during equilibration at a slightly elevated temperature (32 - 34°C) before the start of the run. Reineke (1956) has discussed the relative advantages and limitations of the two types of system.

The detection of steroids on paper is achieved from a knowledge of the behaviour of pure reference substances in the system and by chemical tests with reagents specific for particular groups of steroids. Some steroids are visible on the paper under ultraviolet light. It must be emphasized that no one technique is capable of identifying more than a limited group of compounds, so that chromatographic systems and methods of detection employed vary with the characteristics of the steroids being sought. Bush (1954a) has tabulated appropriate methods of detection and identification for many types of steroid material. More recently Axelrod & Pulliam (1960) have devised six tests for the identification of oestrogen in micro quantities on filter paper and have tabulated the colours produced with each of these tests for eighteen oestrogenic compounds. Three of the tests have also been applied to the detection of sterols.

The mere number of known steroids of closely similar structure and the possibilities for isomerism which exist render identification by chromatographic mobility and reaction to chemical tests inconclusive. The mobility of steroid material is subject to change when non-steroid materials are

present, although the relative positions of the individual steroids with respect to one another is almost always constant (Bush, 1954a). Mobility values in a given system are usually expressed in relation to that of some standard reference compound instead of in relation to distance travelled by the solvent front (Kochakian & Stidworthy, 1952; Savard, 1954). Reineke (1956) has compiled mobility values for a large number of steroids in several different chromatographic systems. Bush (1954a) has pointed out the confusion that may arise from the fact that many of the chemical reagents used for detecting steroids may react with non-steroidal material.

Ultraviolet absorption analysis has been widely applied to the investigation of steroids. Dorfman (1953) published an extensive tabulation of the major ultraviolet absorption peaks of a large number of steroids and steroid derivatives in various organic solvents. Zaffaroni (1950) and Bernstein and Lenhard (1953) studied the ultraviolet absorption spectra of steroids in concentrated sulphuric acid. More recently Bauld et al. (1960) studied the sulphuric acid fluorescence and absorption spectra of eight natural oestrogens, which included the 'classical' oestrogens, 16-epioestriol, 16-oxooestradiol-17β, 16α-hydroxyoestrone, 16-oxooestrone and 2-methoxyoestrone. The spectra of steroids in phosphoric acid has also been investigated (Nowacznski & Steyermark, 1955). Friedgood, Garst & Haagen-Smit (1948) applied ultraviolet

spectrophotometry to the quantitative estimation of oestrone, oestradiol-17β and oestriol. Absorption maxima for all three oestrogens were at 280mμ withasecondary peak at 288 mμ. Beer's Law was obeyed and reproducibility was excellent. However, estimations required 12 to 15μg oestrogen per ml, so that the method is insufficiently sensitive for most urinary assays. Moreover, the method is highly non-specific, because phenols absorb at 270 mμ and consequently any phenolic impurities in urinary fractions may cause marked interference (Bauld & Greenway, 1957). Infrared spectrophotometry has become increasingly useful in providing a great deal of information on the structure of steroid materials (Jones & Herling, 1954). Two volumes of a comprehensive atlas of the infrared spectra of steroids have been published (Dobriner, Katzenellenbogen, & Jones, 1953; Roberts, Gallagher & Jones, 1958). The application of infrared analysis to materials obtainable from paper chromatograms is rather limited due to technical difficulties arising from presence of interfering materials derived from the paper, (Layne, 1957).

The characterization of steroids by the use of mixed chromatograms, can be applied provided that labelled reference samples of the steroids are available. This technique has been described by Roberts et al.(1955) and by Layne et al. (1958) for the identification of oestrone and oestradiol-17β in avian ovarian extracts. The isolated compound and the labelled

reference sample are mixed and chromatographed together. The chromatogram after development is then stained with a chemical reagent which has previously been shown to react in a similar fashion with the reference steroid. A radioautograph of the stained strip is then made. Congruence in position and shape as between the material as located by radioautography and by the staining reaction constitutes strong evidence for the identity of the unknown with the reference substance. The reverse of this technique (chromatography of an unknown radioactive metabolite from urine with a suitable reference compound) has been used successfully by MacRae, Layne & Common (1959), MacRae & Common (1960); and MacRae, Dale & Common (1960) for the identification of radioactive metabolites in urine following injection of oestradiol-17β-16-$C^{14}$ and oestriol-16-$C^{14}$ into the wing vein of a laying hen.

The quantitative assay of isolated oestrogens can be accomplished chiefly by colorimetric, fluorimetric, spectrophotometric and biological methods. The most widely used colorimetric method for the oestrogens is the Kober (1931) reaction which is surprisingly specific for this class of compounds. An excellent review on the development of this reaction is given by Bauld & Greenway (1957). Recently, Ittrich (1958) has modified the Kober reaction by extracting the pink complexes formed with a 2% solution of p-nitrophenol in chloroform containing 1% ethyl alcohol. In a second paper, (Ittrich, 1960a) an alcohol solution of 1% ethylene tetra-

bromide was used to extract the Kober complexes. The extraction of the Kober complexes by organic solvents was further modified (Ittrich, 1960b) by using a mixture of 2% p-nitrophenol and 1% ethanol in ethylene tetra bromide. By using this solvent optimal results were obtained when the colour was measured fluorimetrically. The extraction of the Kober colour minimizes the effect of interfering substances, and the method can easily be applied to routine analysis. Breuer & Gertz (1960) have made an evaluation of the Ittrich procedure and recommended a modification of the extraction procedure for the determination of oestrogens in pregnancy urine. They state that the Ittrich method is not superior to that of Brown (1955) for the colorimetric determination of individual oestrogens present in low concentration in the urine of post-menopausal women and male subjects. In such instances the fluorimetric measurement is preferable. Givner, Bauld & Vagi (1960a, b) have used the Kober reaction to determine quantitatively 2-methoxy-oestrone, oestrone, ringD α-ketolic oestrogens, oestradiol-17β, 16-epioestriol and oestriol after separation by partition chromatography and the Girard reaction.

Kadis & Salhanick (1959) reported a new colourimetric method for the determination of the oestrogens using piperonal chloride in trifluoroacetic acid. The method appears to be considerably more convenient than the Kober reaction in that it requires a single reagent for the three classical oestrogens

and the reaction may be carried out in a much shorter time than that required for the Kober reaction. The authors claim the reaction to be more specific for the oestrogens and more sensitive than the Kober reaction. The chief disadvantages are the necessity for the synthesis of piperonal chloride and the corrosive and volatile characteristics of trifluoroacetic acid.

In recent years, considerable progress has been made in the fluorimetric determination of oestrogens. This method has about one hundred times the sensitivity of the Kober reation. However, in the estimation of oestrogens in urinary extracts, the method is rendered inaccurate by self-absorption caused by impurities which are coloured in hot sulphuric acid. Rigorous purification is required not only for specificity but also for accuracy. Bauld & Greenway (1957) have reviewed the development of this method for the estimation of urinary oestrogens.

# 3. EXPERIMENTAL METHODS

## 3. 1. GENERAL MATERIAL AND APPARATUS

### 3. 1. 1. Hormones and Related Compounds

Crystalline oestrone, oestradiol-17β obtained from Steroid Laboratories Ltd., Montreal, Canada.

Crystalline oestriol, oestradiol-17α obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A.

Crystalline, 16-epioestriol, 17-epioestriol, 16,17-epioestriol, 16-oxooestradiol-17β, 16-oxooestradiol-17α, 16α-hydroxyoestrone, 2-methoxy oestrone, presented by Dr. T.F. Gallagher and Dr. J. Fishman, Sloan-Kettering Institute, New York, U.S.A.

Crystalline equilenin, equol-equol dibenzoate mixture, presented by the late Dr. D.A. McGinty, Parke Davis & Company, Detroit, U.S.A.

Crystalline, equol, equol dibenzoate, equilin, 17-epi-oestriol, presented by Professor Wm. Klyne, Westfield College, London, N.W.3., England.

Crystalline dihydroequilenin-17α, equilin, dihydro-equilin-17α, $\Delta^{5,7,9}$ oestratrienolone and $\Delta^{5,7,9}$-oestratrienol-3-one 17-acetate, presented by Dr. W.L. Glen, Ayerst, McKenna & Harrison, Montreal, Canada.

Crystalline 16-oxooestrone, 2-hydroxyoestradiol-17β, 16β-hydroxyoestrone diacetate, presented by Privat-Dozent Dr. Hans Breuer, Chirurgische Universität-Klinik, Bonn, Germany.

Crystalline 6-dehydroestrone, presented by Dr. Byron Riegel, G.D. Searle & Company, Chicago, U.S.A.

Oestrone-16-$C^{14}$, obtained from Merck & Company, Montreal, Canada.

3. 1. 2. Solvents.

Methanol, 'Analar' reagent; petroleum ether, boiling range 80-100°C, 'Analar' reagent; propylene glycol, laboratory reagent, obtained from British Drug Houses, Ltd.

Chloroform, laboratory reagent, redistilled; n-Heptane, laboratory reagent, obtained from Fisher Scientific Company.

Diethyl ether, U.S.P., Nichols Chemical Company, freed from peroxides according to the procedure of Bauld (1956) and stored over a zinc-copper couple.

Benzene, A.C.S. reagent (thiophene-free), obtained from Anachaemia.

Skellysolve-C (essentially n-Heptane), boiling range 85 - 100°C, obtained from Skelly Oil Company.

Dichloromethane, obtained from Eastman Organic Chemicals.

Formamide, laboratory reagent, obtained from Fisher

Scientific Company, purified for chromatographic use according to the procedure of Cole, Knox & Picchioni (1959).

3. 1. 3. Colour Reagent

Diazotised sulphanilic acid, prepared immediately before use according to the directions of Block & Bolling (1951).

3. 1. 4. Enzyme Preparation

The enzyme preparation used is known commercially as 'Glusulase' and purchased from Endo Laboratories, Inc., Richmond Hill, New York, U.S.A. Each cc. of the preparation is standardized to contain 100,000 Fishman Units of β-glucuronidase and 50,000 Fishman Units of Sulphatase.

3. 1. 5. Chromatographic Apparatus.

Four chromatographic systems have been used in this work and these will henceforeward be referred to as System A, B, C & D, respectively.

System A consisted of petroleum ether, 'Analar' reagent (B.D.H.), free from aromatic hydrocarbons (boiling range 80-100°C), as the mobile phase and absolute methanol as the stationary phase. This system is essentially that described by Bush (1952) and by Layne et al. (1958).

The chromatographic apparatus used for this system has

also been described previously by Layne (1957). Whatman No. 42 filter paper strips were used. The width of strip used varied depending on the nature and the amount of material being chromatographed. The paper strips were prepared for chromatography by continuous extraction with petroleum ether and methanol (1:1 $^{v}/_{v}$) in a Soxhlet apparatus for 24 hours. Immediately before chromatography the paper strips were dried in an air oven at 100°C. The material to be separated was applied to the strip at a point 8 cm. from one end of the strip. Saturation of the chromatographic chamber was accomplished by filling the bottom of the apparatus to a depth of about 3 cms. with the stationary phase and placing a beaker containing 300-500 ml. of the mobile phase in the bottom of the chamber. The entire apparatus was placed in an insulated cabinet maintained at 34°C. The cabinet was provided with a glass inner door so that the progress of a run could be checked without altering the temperature. Before development of the chromatogram (s), the whole apparatus was equilibrated at 34°C overnight. Development of the chromatogram usually required 4 - 5 hours.

System B was that described by Layne & Marrian (1958), using chloroform (redistilled) as the mobile phase and formamide (purified as described previously) as the stationary phase. The chromatographic apparatus used for this system and for system D consisted of a glass chromatographic tank

with a glass solvent trough held in position by glass supports, which were carried in stainless steel brackets screwed to opposite sides of the tank. (This piece of equipment was obtained from Shandon Scientific Company, London, S.W.7., England.)

Chromatography was carried out using Whatman No. 42 Filter Paper. The paper strips were prepared for chromatography by thorough extraction in a Soxhlet apparatus with a mixture of methanol and chloroform (1:1 v/v) for three days. Before a chromatographic run, the paper strips were impregnated with the stationary phase by immersing in a 2:1 (v/v) methanol-formamide mixture, blotted with filter paper between two sheets of plate glass and dried in a horizontal position for 45 minutes. The chromatographic runs were carried out at room temperature. Saturation of the chromatographic chamber was accomplished by filling the bottom of the tank to a depth of about 1 inch with the mobile phase (previously saturated with formamide). The whole apparatus was equilibrated at room temperature for about 1 hour before the development of the chromatogram (s). Development of the chromatograms usually required 6 - 8 hours under these conditions.

System C was that described by Bush (1952) and by Breuer, Knuppen & Pangels (1959). The mobile phase was benzene (thiophene free) and the stationary phase was a mixture of methanol and water. The final ratio benzene: methanol: water

was 100:55:45 by volume. The chromatographic apparatus was that used for system A. Chromatography was carried out using Whatman No. 42 filter paper. The filter paper strips were prepared for chromatography by continuous extraction in a Soxhlet apparatus with a mixture of benzene and methanol (1:1$^{v}$/v) for 24 hours. Saturation of the chromatographic chamber was accomplished as for system A. The chromatographic runs were carried out at a temperature of 30°C and not at 34°C as described by Bush (1952). This lower development temperature extends the time of the chromatographic run by about 3 hours to give a running time of 6 - 8 hours. The whole apparatus was equilibrated at 30°C for about 12 hours before development of the chromatogram (s).

System D was that described by Gorski & Erb (1959) for the separation of oestradiol-17β and oestradiol-17α. Skellysolve C was used as the mobile phase and formamide (purified as described previously) as the stationary phase. Whatman No. 42 filter paper strips were used and subjected to preliminary extraction with Skellysolve C and methanol (1:1$^{v}$/v) as described previously for system B. Prior to a chromatographic run the strips were impregnated with formamide as described previously for system B. The chromatographic chamber was similar to the one used in system B. Saturation of the chamber was accomplished by covering the bottom of the chamber to a depth of about 1 inch with the mobile phase previously saturated with formamide. The chromatograms were developed at room temperature

after equilibration of the apparatus at room temperature with the mobile phase saturated with formamide for 12 hours. In order to achieve a good separation of the material on the chromatogram, the chromatogram was developed for 24 hours.

In all four systems the material to be separated was applied across the width of the paper in a strip about 1/2 cm. wide at a point 8 cms. from one end of the paper.

3. 1. 6. Measurement of radioactivity

The apparatus used throughout the present work consisted of a Forro Chromatogram Scanner (purchased from the Forro Scientific Company, Evanston, Illinois, U.S.A.) which is designed to scan paper chromatograms tagged with low energy beta emitters, such as carbon 14, sulphur 35, calcium 45, tritium. In the present work, it was desired to count $C^{14}$. The detecter is a small, unprotected, gas flow counter, operating in the Geiger region, with a background of 15 c.p.m. The scanning head is provided with two different slits (1/16 inch and 1/4 inch in width). The scanner is used with a thin window, the window having a surface density of about 0.8 mg. per $cm^2$. Since there is no gap between the paper strip and the active volume of the counter, the efficiency is high. The detector is connected to a rate meter which feeds an Esterline-Angus automatic recorder (Esterline-Angus Co. Inc. Indianapolis, Indiana, U.S.A.). The recorder inscribes the

count rate in its true shape on the chart. Chart and chromatogram speeds are identical, which facilitates evaluation of the results by simple alignment of the chromatogram with the recorder chart. A simple setting of a lever allows instant selection of ten different scanning speeds, ranging from 0.75 inch per hour to 12 inch per min. The scanning is automatically interrupted when reaching the end of the chromatogram and a sound signal given to alert the operator. The rate meter used was a Baird-Atomic Research Ratemeter, Model 432A (Baird Atomic Inc., Cambridge, Mass., U.S.A.).

## 3. 2. EXPERIMENTAL METHODS

### 3. 2. 1. Hydrolysis and extraction of oestrogens from urine and faeces.

It is well known (see above) that acid hydrolysis leads to some destruction of the 'classical' oestrogens and to complete destruction of the 16-hydroxyoestrogens and of 16-oxo-oestrone. Accordingly, enzymatic hydrolysis was used almost exclusively in the present work. The technique used was that of Layne & Marrian (1958), except for the use of a bacterial β-glucuronidase and sulphatase enzyme preparation ('Glusulase') instead of the limpet powder used by Layne & Marrian (1958). In cases where enzymatic hydrolysis was not used, the hydrolysis and extraction procedure used was the method of Bauld outlined by Bauld & Greenway (1957). Since the latter method was used only once in the present work, it will be outlined in the

appropriate section under experimental procedures. The procedure of Layne & Marrian (1958) has been adapted to the extraction of oestrogens from both urine and faeces; a detailed outline of the methods is given below.

3. 2. 2. Hydrolysis and extraction of oestrogens from urine.

The urine sample (usually about 300 - 500 ml.) for 24 hours was adjusted to pH 4.8±0.1 by the addition of acetic acid followed by addition of a volume of molar acetate buffer equol to one-fifth of the volume of the urine. 'Glusulase' was added at the rate of 500 Fishman units per ml. urine and the mixture was incubated at 37°C for 24 hours. Before extraction with an equal volume of ether, Bradosol (a 5% solution of β-phenoxyethyldimethyldodecylammonium bromide, manufactured by Ciba Ltd.)(1ml/l.) was added to facilitate the breaking of emulsions. The ethereal extract of the enzymatic digest was washed four times with a 5% solution of sodium bicarbonate and twice with water, dried over sodium sulphate and evaporated to dryness. The residue after evaporation was taken up in methanol for chromatography.

In the procedure of Layne & Marrian (1958), the residue after evaporation of the ether is taken up in ethanol and subjected to a Girard separation to effect further purification. In the present work further purification by the Girard separation was found to be unnecessary.

3. 2. 3. Hydrolysis and extraction of oestrogens from faeces.

The faeces were dispersed in water (300 ml.) and to the mixture was added an equal volume of methanol. This mixture was refluxed for 20 minutes and filtered with suction. The residue was extracted under reflux with absolute methanol for a further 20 minutes, filtered and the filtrate added to the first filtrate. The combined filtrates were evaporated in a rotary evaporator under reduced pressure until water and methanol had been removed. The residue was taken up in 500 ml. water (dark green solution), and subjected to hydrolysis and extraction by the procedure of Layne & Marrian (1958) outlined above. After evaporation of the ether the residue was taken up in 100 ml. of 70% ($^{v}/v$) aqueous methanol. Cholesterol, gummy impurities and most of the green colouration were removed from this solution by two washings each with an equal volume of n-heptane, as recommended by Breuer & Knuppen (1960a). (This procedure was found extremely useful for faecal extracts.) The aqueous methanolic solution was evaporated to dryness under reduced pressure and the residue was taken up in methanol for chromatography.

3. 2. 4. Preparation of the final extracts for chromatography.

The final extracts, obtained by the various procedures, were prepared for chromatography by first reducing the solvent to about 1 ml. in a 50 ml. round bottom flask. The flask was

rinsed with four successive 2 ml. volumes of a methanol dichloromethane mixture (1:1 v/v). The washings were transferred with a dropper to a 15 ml. conical centrifuge tube, and evaporated down to a few drops under reduced pressure. The walls of the tube were washed down with a further 0.5 ml. of dichloromethane-methanol and the solution in the bottom of the tube again evaporated down to a few drops. This solution was applied to the paper chromatographic strips with a 50 microlitre pipette. The tube was washed once with a few drops of solvent and these washings applied to the paper strip.

When extracts were being chromatographed, the material was applied in narrow horizontal bands across the width of the paper strip. It was possible, therefore, to cut narrow strips from the sides of the chromatogram. These narrow strips could be stained with certain colour reagents in order to locate the components. When reference compounds were chromatographed, either alone or with extracts, the material was applied as spots.

When it was decided to elute a particular portion of the chromatogram in order to subject it to further chromatography, the relevant portion was cut from the paper strip and cut into small pieces with a sharp pair of stainless steel scissors. These pieces were completely immersed in methanol-dichloromethane and left for five hours. The solvent was then transferred to a 15 ml. centrifuge tube. The paper was extracted a second time with the same solvent, and this

extract was then added to the first eluate. The combined eluate was then evaporated down to a few drops under reduced pressure and the material applied to a paper chromatographic strip as previously described.

3. 2. 5. Chromatography

System B was used throughout the present work as a means of separating the oestrogenic material after extraction either from urine or faeces. This system was used by Layne & Marrian (1958) to separate the α-ketolic oestrogens. As will be seen in the experimental procedures and results, this system is also capable of separating the four isomers of oestriol, 16-oxooestrone, oestradiol-17β and oestrone. Systems A & C were used only as a means of identifying or confirming the material separated in system B. System D was used for the purpose of achieving a separation between oestradiol-17β and oestradiol-17α. For all four systems, before the chromatogram was developed the whole apparatus was allowed to equiliberate at the temperature of development, for the times indicated previously, before the run was made. At the start of a run 30 ml. of the mobile solvent was rapidly poured into the trough through a funnel, the stopper sealing the apparatus being immediately replaced to prevent desaturation of the atmosphere in the chamber. In the case when either systems A or C were used, the outer door of the constant temperature cabinet was only opened for short periods for occasional observation. Care

was taken in all experiments to avoid exposure of the chromatograms bearing steroid material to strong light, and in particular to fluorescent lighting.

Figure 4 shows typical separations obtained with solvent systems A & B. The $R_F$ values are reproducible with pure steroids, but the non-steroidal material present in extracts from tissues caused changes in the mobility of the steroids. This was particularly true for system A.

System B gave better reproducibility of $R_F$ values even in the presence of contaminating materials. After several chromatographic purifications, the steroids obtained from extracts usually attained the same mobilities as the reference compounds. In order to obtain reproducible results in system A, it is essential to dry the paper strips in an air oven at 100°C for at least 20 minutes before applying the material to be separated. The poor separation of the oestrogen in system A consequent on the presence of hygroscopic moisture was first pointed out by Layne (1957). The $R_F$ values of some of the reference oestrogens used in the present work, when run in the chloroform-formamide system (system B) are illustrated in Table 1.

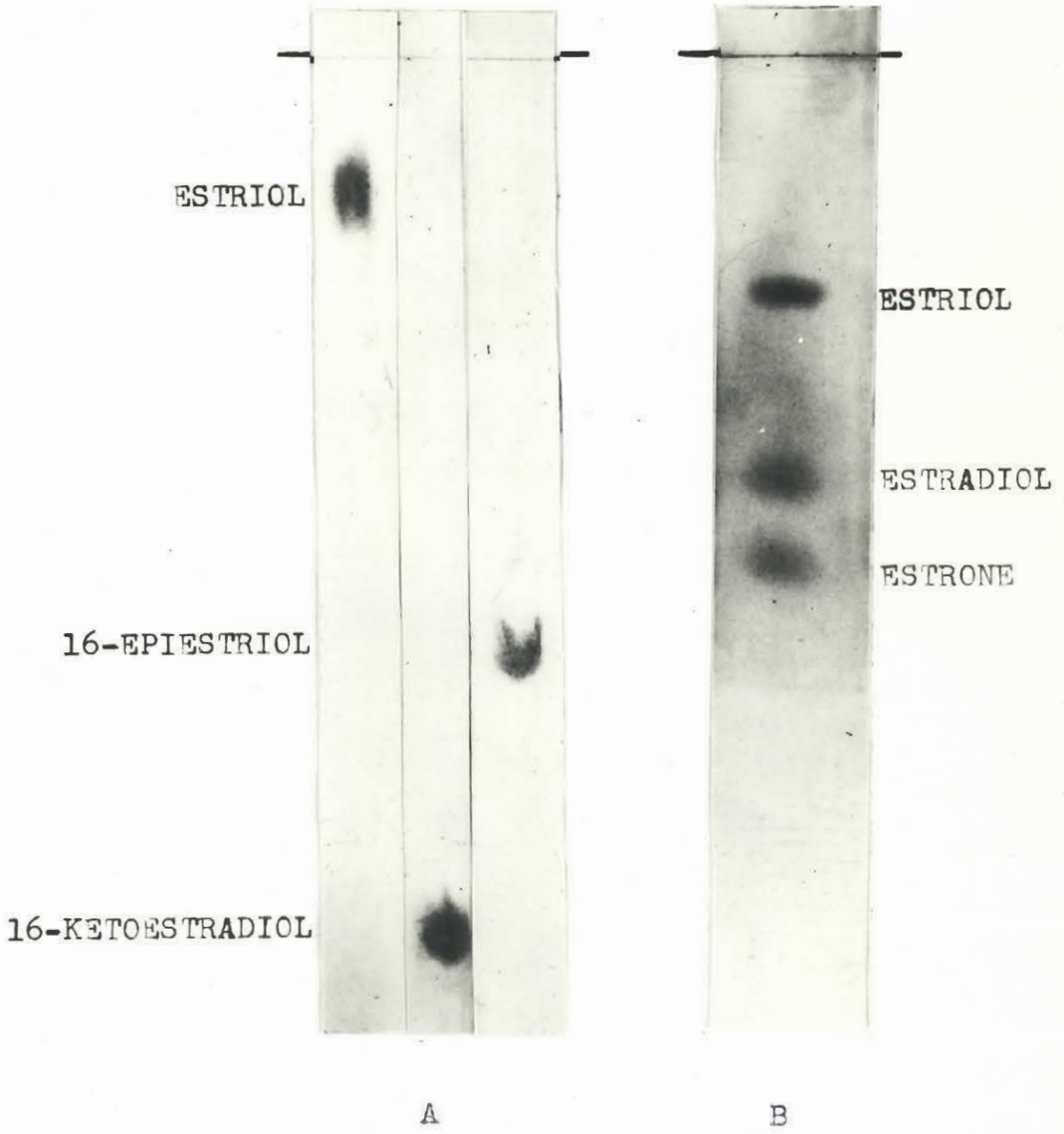



FIGURE 4. Photocopies of typical chromatograms showing separations obtained with reference steroids.

A. Oestrogen spots on chromatograms developed in System B for 6.5 hours and stained with DSA.

B. Oestrogen spots on chromatograms developed in System A for 10 hours and stained with DSA.

TABLE 1

| Reference material | $R_F$ value |
|---|---|
| Oestriol | 0.046 |
| 16-epioestriol | 0.27 |
| 16β-hydroxyoestrone | 0.44 |
| 16-oxooestradiol-17β | 0.51 |
| 16α-hydroxyoestrone | 0.57 |
| Oestradiol-17β | 0.64 |
| Oestradiol-17α | 0.66 |
| Equilenin | 0.82 |
| Equilin | 0.84 |
| 6-dehydrooestrone | 0.85 |
| Oestrone | 0.90 |
| 2-methoxyoestrone | 0.93 |

$R_F$ values of some reference oestrogens when run in the chloroform-formamide system for 6 - 7 hours under the stated conditions.

3. 2. 6. Detection of steroids on paper.

The position of the oestrogens on paper strips was established by cutting a strip, 2 to 3 mm. wide, from the edge of the chromatogram and then treating with the colour reagent. Henceforeward, in this thesis the expression "stained with DSA" will imply the immersion of the chromatogram in the diazotised sulphanilic acid reagent, followed by immersion in sodium carbonate until the full colour has developed. The colour of the spots or bands varied between yellow and orange-yellow depending on the oestrogen or oestrogens present on the strip. The spots or bands could be stabilized by re-immersing the strip in the diazotised sulphanilic acid. The only disadvantage of re-immersing in the diazotised sulphanilic acid is that the intensity of the spot is diminished somewhat. The presence of the ring B unsaturated oestrogens (equilin, equilenin and 6-dehydrooestrone) can be detected immediately, since they give a distinct red colour when stained with DSA. This reagent was capable of detecting the oestrogens in concentrations as low as 5 micrograms per sq. cm. of paper.

Since the oestrogens present on the chromatograms throughout this work were radioactive, a simple method of detection was to scan the chromatographic strip using the Forro Radio Chromatogram Scanner. The position of the radio-active material on the paper could be determined by the simple alignment of the recording on the chart with the

chromatographic strip. The limits of the radioactive zones could then be marked with a pencil.

3. 2. 7. Measurement of radioactivity.

The radioactivity present on the chromatograms was measured by cutting the chromatograms longitudinally into strips each approximately 1/2 inch in width. Each strip was then counted on the Forro Radio Chromatogram Scanner. The total counts on a strip or segment thereof were measured by planimetry of the areas under the curves. Planimetry was carried out on the curves after their transformation to rectangular coordinates, although planimetry on the tracings on the charts themselves is satisfactory for most purposes.

To obtain reproducible results, it is essential that the geometry of counting should be the same between measurements, i.e. the width of the strip being counted, the time constant, the speed at which the chart moves, the width of the slit over which the strip moves and the voltage should be the same, for each strip being counted.

Throughout this work the radioactivity was measured using the following constants:-

| | | |
|---|---|---|
| Time Constant | = | 100 sec. |
| Chart Speed | = | 1 1/2 inch per hour. |
| Slit Width | = | 1/16 inch |
| Width of Strip | = | 1/2 inch |
| Voltage | = | 1080 v. |

The full scale reading varied according to the amount of radioactivity present on the strip.

3. 2. 8. Identification of steroids by radioautography.

The technique of running chromatograms of an unknown steroid with a radioactive reference steroid can be used to provide a final identification of steroids (Layne, Common, Maw & Fraps, 1958). The spot to be tested is eluted from the chromatogram and mixed with 25 - 30 micrograms of the appropriate pure steroid labelled with $C^{14}$. The mixture is chromatographed and then stained with DSA. The stained chromatogram is then photographed and then placed on X-ray film in a darkroom. After a certain period of exposure, the film is developed and the position of the dark spot caused by the radioactive material is recorded by photography. A positive identification hinges on a complete correspondence in detail between the position and shape of the spot so indicated by the radioautograph with the position and shape indicated by the DSA stain.

The reverse of this technique has been used in the present work to identify radioactive oestrogen metabolites isolated from avian excreta following the injection of oestrogens labelled with $C^{14}$. In this method, the radioactive spot (insufficient to give a perceptible staining with DSA) was eluted from the chromatogram and mixed with 20-25 micrograms of the appropriate non-labelled oestrogen. The mixture was then chromatographed on a paper strip and stained with DSA. The stained chromatogram was photographed and then

placed on X-ray film for exposure. The exposure time depended on the amount of radioactive metabolite present and had to be established by trial in each case. The X-ray film was developed and the position of the dark spot was recorded by photography. A positive identification depends again on a complete correspondence in detail between the position and shape of the spot as indicated by the radioautograph and as indicated by the staining reaction. For the conclusion to be valid, the amount of labelled compound isolated should be insufficient to give perceptible staining with DSA, so that the perceptible staining reaction of the mixture can be considered attributable solely to the added non-active carrier.

3. 2. 9. Identification of steroids by reduction with sodium borohydride.

A method which has been found very useful in the identification of steroids is by reduction of the ketone group at C-16 or C-17 with sodium borohydride. Using this technique, Biel (1951) has shown that oestrone yields oestradiol-17β exclusively. It is expected that oestriol and 16-epioestriol are formed respectively when 16α-hydroxy-oestrone or 16β-hydroxyoestrone are reduced, depending on the configuration of the C-16 hydroxyl group. On the other hand 16-oxooestradiol-17β can give rise to 16-epioestriol on sodium borohydride reduction, as shown by Huffman & Lott (1955)

in which they obtained 16-epioestriol in almost quantitative yield by this procedure.

In many cases the reduced steroid yields a mixture of products which have to be separated before comparison with the products obtained by the reduction of a suitable reference steroid. Identification is based on comparison of the melting point or melting points of the reduction products with those of the reference steroid and by comparison with the mixed melting point.

In the present work this method of reduction has been applied to the identification of radioactive metabolites obtained from avian excreta after injection of $C^{14}$ labelled oestrogens. To the unknown metabolite is added about 50 $\mu$gms. of the appropriate reference substance. This is then reduced with sodium borohydride by the method described below. The reduced compounds are extracted and chromatographed. The chromatogram is then stained with DSA and photographed, and it is then placed on X-ray film in a dark room. After an appropriate period of exposure, the film is developed and the position of the dark spot or spots caused by the radioactive material is recorded by photography. Positive identification is based on a complete correspondence in detail between the position and shape of the spot or spots as indicated by the radioautograph with the position and shape indicated by the DSA stain. In this way congruence of staining as regards

shape and position of the chromatogram with the position and shape of the spots of the radioautogram shows that the reference steroid and the radioactive metabolite are almost certainly identical.

The following method was used to reduce the material with sodium borohydride. The material to be reduced was dissolved in ethanol and diluted with water (ethanol concentration of final solution is about 50%). Sodium borohydride (obtained from L. Light & Co., Ltd., Colnbrook, England) was added to the solution at the rate of 10 mgm. per ml. The tube (or flask) was shaken intermittently by hand and allowed to stand overnight in the dark. The tube or flask should be stopperedvery loosely, since there may be considerable evolution of gas. The excess borohydride present was then destroyed by the careful, dropwise (effervescence) addition of 10% (v/v) acetic acid. The reduced compounds were then extracted with ethylene dichloride or other suitable solvent and this was subsequently freed from acid by shaking with 8.5% sodium bicarbonate solution. If ether is used for extraction, considerable washing with bicarbonate is required, since ether dissolves considerable amounts of acetic acid.

## 4. EXPERIMENTAL PROCEDURES AND RESULTS

Studies on the metabolism of oestrone-16-$C^{14}$ *in vivo* by the laying hen.

### 4. 1. EXPERIMENT 1.

#### 4. 1. 1. Object

If the metabolic pathway of oestrogens in the laying hen is generally similar to that of humans, (see Fig. 3.) then the biogenesis of oestrone would proceed via the pathways indicated. In view of this it seemed desirable to establish whether or not the biogenesis of oestrogen in the fowl follows this scheme.

This experiment was designed, therefore, to identify the conversion products of injected radioactive oestrone in the laying hen. Since studies on the droppings are complicated by the possibility of secondary changes due to bacterial action (MacRae, Dale and Common, 1959), it was considered necessary to examine the urine and faeces separately. Special attention was given to the possible occurrence of 16-oxooestrone as a conversion product of oestrone in view of the results reported by Slaunwhite and Sandberg (1956) for the human subject. Attention was also given to the possible occurrence of 16, 17-*epi*oestriol, 17-*epi*oestriol and the 2-methoxyoestrogens as conversion products of oestrone in the fowl.

4. 1. 2. Method.

The urine and faeces of birds are voided into the cloaca. In order to obtain urine free from faecal matter, it is necessary to cannulate the ureters or surgically to exteriorise the openings of the ureters or of the rectum.

In the present work the ureters of the experimental hens were exteriorised by the operative procedure of Dixon and Wilkinson (1957). This operation can be performed quite rapidly and, once healing has occurred, the bird may be used indefinitely. The main surgical problem encountered was that the birds developed fistulas, usually about one week after the operation. The fistulas developed in the region where the mucosa containing the uretal openings was separated from the mucosa of the cloaca. The operated birds were kept in ordinary large individual cages where they were free to move about, and in this environment they soon came into lay. The birds were fitted with a canvas harness which was used to hold the urine collection tubes in place. The urine was collected in latex rubber tubes (artificial vagina reliners) which were sutured to the upper lip of the vent. With this arrangement, it was merely necessary to tie the lower end of the collection tube over the back of the bird. Urine collections could be made readily at frequent intervals throughout the day.

Birds with exteriorised ureters required considerable care because the urine was quite concentrated and abundant in urates. The latter tended to collect in the region of the exteriorised ureters, and had to be washed off each time urine was collected. The washings resulting from this were always added to the urine sample. If urates are allowed to accumulate in this region, the tiny uretral openings may become blocked and if this is allowed to occur, the bird will die usually within twenty-four hours.

In this experiment, ten microcuries (0.233 mg.) oestrone-16-$C^{14}$ in 2 ml. propylene glycol was injected into the wing vein of a White Rock laying hen on the afternoon of the 45th day after the operation for exteriorised ureters. The urine of the succeeding 24-hour period was collected and worked up for oestrogens by the method described in Section 3. 2. 2. The final extract was chromatographed in System B. After development, the chromatogram was exposed to X-ray film for 30 hours.

4. 1. 3. Results and Discussion.

The radioautogram of the chromatogram revealed twelve radioactive zones. An equol zone was located by cutting a narrow longitudinal strip from the main chromatogram and staining with D.S.A. Equol was first discovered by Marrian and Haslewood (1932b) who isolated it from mare's

pregnancy urine. It has been found in the urine of the cow (Klyne and Wright, 1956) and of the goat (Klyne and Wright, 1957). Equol is thought to be a metabolite of plant isoflavones; its occurrence in the excreta of the fowl would be consistent with the fact that the diet was a commercial feedstuff mixture designed for laying hens and contained dried alfalfa meal. Its presence calls for close attention in studies on oestrogens in the fowl, since, as will be described later in this discussion, it can give rise to imperfect resolution of the chromatograms. None of the oestrogens were present in amounts sufficient to give a perceptible stain with DSA. The main strip was then divided into a part carrying the four less polar zones and another carrying the remaining zones. Each part was eluted separately. The eluates were rechromatographed separately in System B and radioautographed with the results shown in Fig. 5. The various zones were numbered for convenience of reference.

Figure 5 shows that the zones were distinct and well separated. Attempts were now made to identify the zones by eluting each radioactive zone from the chromatograms and chromatographing the eluates with appropriate reference compounds. The technique used has been described previously in Section 3. 2. 8.

FIGURE 5. Radioautograms of chromatograms (chloroform-formamide system (A) more 'polar' and (B) less 'polar' urinary oestrogens of the laying hen after injection of oestrone-16-$C^{14}$.

0. Zone of application.

1. Oestriol.

2. Mainly 16-epioestriol with some oestriol (see text).

3. 16-epioestriol

4. 17-epioestriol

5. Not identified.

6. 16-oxooestradiol-17β.

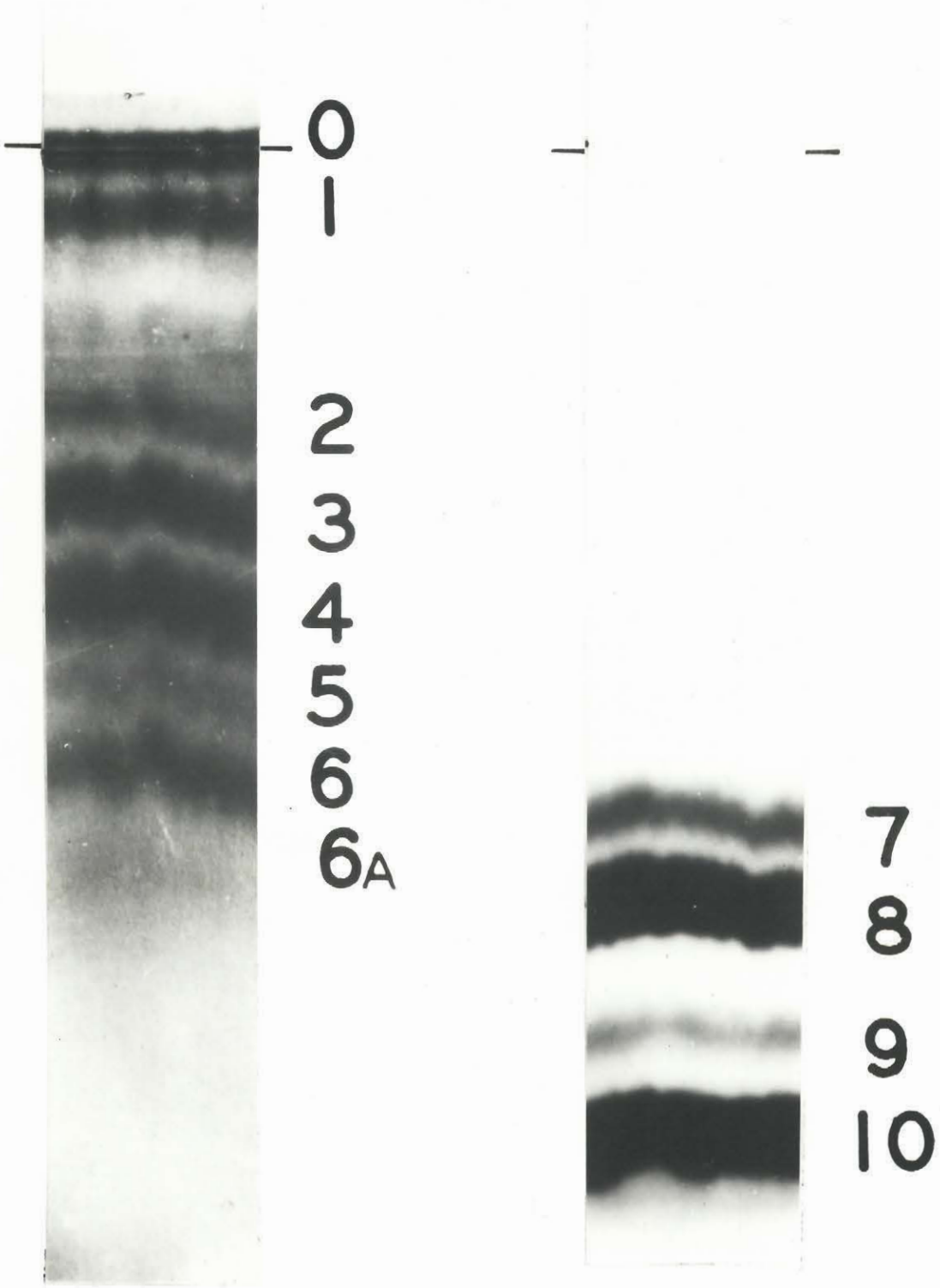
6A. Not identified.

7. Oestradiol-17β.

8. 16-oxooestrone.

9. Not identified.

10. Oestrone.


0
1
2
3
4
5
6
6A
7
8
9
10
A
B

Zone 1 was eluted, mixed with 25 micrograms reference oestriol, rechromatographed in System B, stained with DSA and radioautographed. Congruence of staining and blackening of the X-ray film confirmed the tentative identification of this zone as oestriol.

Zone 3 was tentatively identified as 16-epioestriol by a similar chromatographic comparison with reference 16-epioestriol.

Zone 6 was tentatively identified as 16-oxooestradiol-17β by a similar comparison with reference 16-oxooestradiol-17β.

Zone 10 was tentatively identified as oestrone by a similar comparison with reference oestrone. The radioautograms afforded no indication of the presence of any radioactive metabolite less 'polar' than oestrone. This fact was significant in that it suggested that 2-methoxy-oestrone was absent, and this in turn suggested that 2-methoxy- or 2-hydroxyoestrogens might not be significant as urinary metabolites of oestrone in the laying hen.

All the foregoing oestrogens had previously been detected in extracts of avian excreta (Hurst, Kuksis and Bendell, 1957; MacRae, Layne and Common, 1959; MacRae and

Common, 1960; MacRae, Dale and Common, 1960).

Preliminary inspection of the radioautogram suggested that Zone 2, as shown in Fig. 4, might either represent more than one component or be due to imperfect chromatographic resolution caused by the presence of contaminants. Accordingly, Zones, 1, 2 and 3 were eluted from part of the chromatogram and rechromatographed in System C. A single, broad radioactive zone was found on the resultant radioautogram. This zone was eluted and chromatographed again in System B. This time there were <u>two</u> distinct radioactive zones. The more polar of these was tentatively identified as oestriol and the less polar as 16-<u>epi</u>oestriol by appropriate mixed chromatograms. These observations, which are recorded in Fig. 6, supported the conclusion that Zone 2 was due to contaminants, e.g. equol, which interfered with the resolution of oestriol and 16-<u>epi</u>oestriol. The greater part of the activity in Zone 2 was almost certainly due to 16-<u>epi</u>oestriol. In subsequent work (Section 4. 3. 2.) this imperfect resolution in System B was largely overcome.

Attention was now directed to Zones 7 and 8. It was suspected that one of these zones might be oestradiol-17β since the conversion of oestradiol-17β to oestrone has been shown to be reversible in a number of mammalian species,

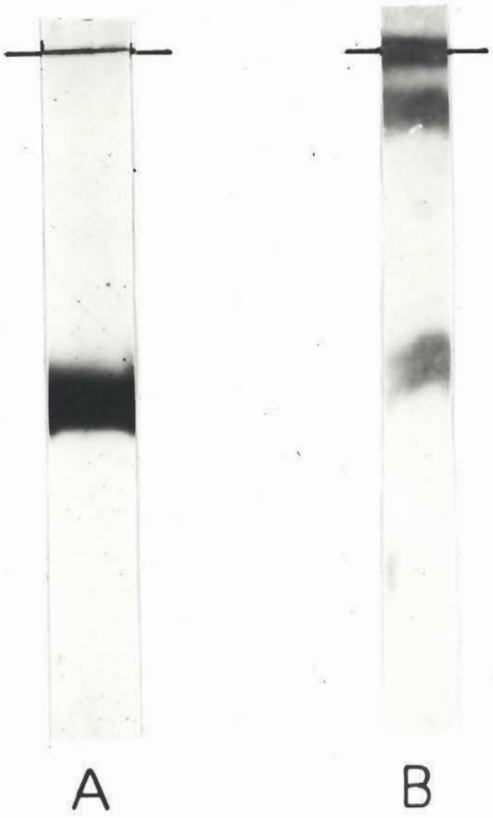



FIGURE 6. Radioautograms of chromatograms of Zones 1, 2 and 3 after elution from original chromatogram (see Fig. 5) and further chromatography in (A) system A and (B) eluate from A in system B.

Note the separation of the eluate from (A) into two zones, which have been shown by appropriate mixed chromatograms to be identical with oestriol (more 'polar' zone) and 16-epioestriol (less 'polar' zone). This indicates that Zone 2 was almost certainly due to the holding-back of radioactivity by non-radioactive contaminants, e.g. equol.

including the hen (MacRae, Layne and Common, 1959). Also, there was the possibility that one of these zones might correspond to 16-oxooestrone, since Slaunwhite and Sandberg (1956) obtained this compound as a major conversion product of oestrone-16-$C^{14}$ when this was injected into a woman in the luteal phase of the menstrual cycle. By running mixed chromatograms of parts of Zone 8 with appropriate reference samples, it was shown that Zone 8 was neither oestradiol-17β nor oestradiol-17α. In view of these negative results the remainder of Zone 8 was eluted with methanol, mixed with 25 micrograms reference 16-oxooestrone and rechromatographed in System C. There was complete congruence of staining and radioautographic blackening of the chromatogram. Breuer, Knuppen and Pangels (1959) have reported that 16-oxooestrone streaks badly in the chloroform-formamide system. For this reason they have advocated preliminary separation of 16-oxo-oestrone by chromatography in System C before attempting separation of the other oestrogens in System B. 16-oxo-oestrone does not streak in System C. These results of Breuer, Knuppen and Pangels (1959) have been confirmed in so far as 25 micrograms of 16-oxooestrone streaked badly in System B, but ran cleanly in System C. The separation of 16-oxooestrone on the chromatograms of urinary extracts in System B, however, was remarkably clean. It is the author's

view that this is almost certainly attributable to the very small absolute amounts of 16-oxooestrone present on the chromatograms. The identification of Zone 8 as 16-oxooestrone strongly indicated that Zone 7 may correspond to oestradiol-17β. On elution, admixture with 25 micrograms reference oestradiol-17β, rechromatography in System B, staining with DSA and radioautography, there was congruence of stain and radioautogram. This confirmed the identity of Zone 7 as oestradiol-17β. Examination for the possible presence of oestradiol-17α by the appropriate chromatography of Zone 7 in System D afforded no evidence for the presence of oestradiol-17α.

It was suspected that Zone 4 might be 17-epioestriol because this oestrogen is slightly less 'polar' than 16-epioestriol and because 17-epioestriol has been isolated as a normal metabolite from human pregnancy urine (Breuer, 1960). Zone 4 was eluted, mixed with 25 micrograms reference 17-epioestriol, rechromatographed in System C, stained with DSA and radioautographed. Congruence of staining and radioautographic blackening confirmed the tentative identification as 17-epioestriol.

Zone 9 represented a metabolite that was slightly but definitely less 'polar' than 16-oxooestrone, but more 'polar'

than oestrone in System B. Professor G.F. Marrian having drawn our attention to the fact that equilin, equilenin and 6-deydrooestrone are all slightly more 'polar' than oestrone in this system, a mixed chromatogram of Zone 9 with 25 micrograms of equilenin was run. There was non-congruence of staining and radioautographic blackening, the reference equilenin being slightly more 'polar' than the radioactive zone. Therefore, Zone 9 is not equilenin, neither is it equilin since equilin and equilenin are not separable in System B. A further attempt was made to identify Zone 9 by running a mixed chromatogram of Zone 9 and 25 micrograms of 6-dehydrooestrone. In this case there was non-congruence of stain and radioautogram and 6-dehydrooestrone was slightly less 'polar' than the radioactive zone. Zone 9, therefore, has not yet been identified.

Zones 5 and 6A were not identified. A mixed chromatogram of Zone 5 with reference 16β-hydroxyoestrone (obtained by hydrolysis of 16β-hydroxyoestrone diacetate). The method of hydrolysis was as follows: 60 micrograms of 16β-hydroxyoestrone diacetate was saponified by treatment with 1 ml. of 5N methanolic sulphuric acid at room temperature for twenty-two hours. The saponified material was then diluted to 5 ml. by the careful addition of iced water.

The mixture was then extracted twice with 5 ml. ether. The ether extract was washed four times with 5 ml. of 5% sodium bicarbonate and twice with water; then dried over sodium sulphate and evaporated to dryness. When the residue was chromatographed in System B with carrier 16β-hydroxyoestrone, there was non-congruence of stain and radioautogram. The reference 16β-hydroxyoestrone was slightly less 'polar' than the radioactive zone. Similarly, a mixed chromatogram of band 6A with reference 16α-hydroxyoestrone showed non-congruence of stain and radioautogram. In this case the reference 16α-hydroxyoestrone was slightly more 'polar' than the radioactive zone.

The tentative identification of the various zones was further confirmed by chromatography of the radioactive zone with the appropriate reference compound in System A. In all cases the positive results as claimed above were confirmed. The results of chromatography of the different radioactive zones with the appropriate reference compounds, indicating the DSA stain and the corresponding radioautogram are shown in Fig. 7.

The foregoing results have shown that oestrone is converted, by the laying hen <u>in vivo</u>, in essentially the same manner as by the human. The identification of 16-oxooestrone as a major conversion product of oestrone in

FIGURE 7. (A) Chromatographic identification of Zones 3, 4, 6, 7, 8 and 10, from the urinary extract of laying hen after injection of oestrone-16-$C^{14}$ and preliminary separation in system B (see Fig. 5).

1A.R. Radioautogram of chromatogram of presumptive 16-epi-oestriol zone after admixture with 25 micrograms of reference 16-epioestriol and chromatography in system B.

1A.S. DSA stain of chromatogram.

1B.R. Radioautogram of a similar mixed chromatogram after chromatography in system A.

1B.S. DSA stain of chromatogram.

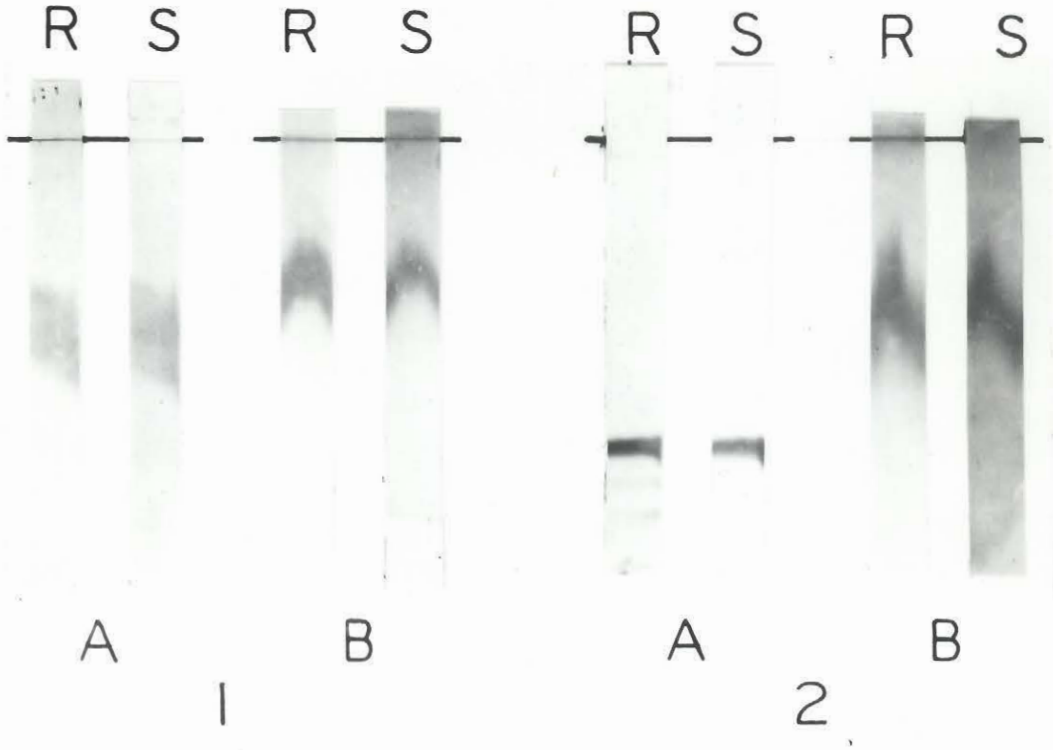
2A.R. Radioautogram of chromatogram of presumptive 17-epioestriol zone after admixture with 25 micrograms of reference 17-epioestriol and chromatography in system C.

2A.S. DSA stain of chromatogram.

2B.R. Radioautogram of similar mixed chromatogram after chromatography in system A.

2B.S. DSA stain of chromatogram.

Note: It was not possible to obtain satisfactory photographic reproductions for the presumptive oestriol zone although by similar chromatography in systems B and A congruence of stain and radioautographic blackening was obtained.


R S R S R S R S
A B A B
1 2

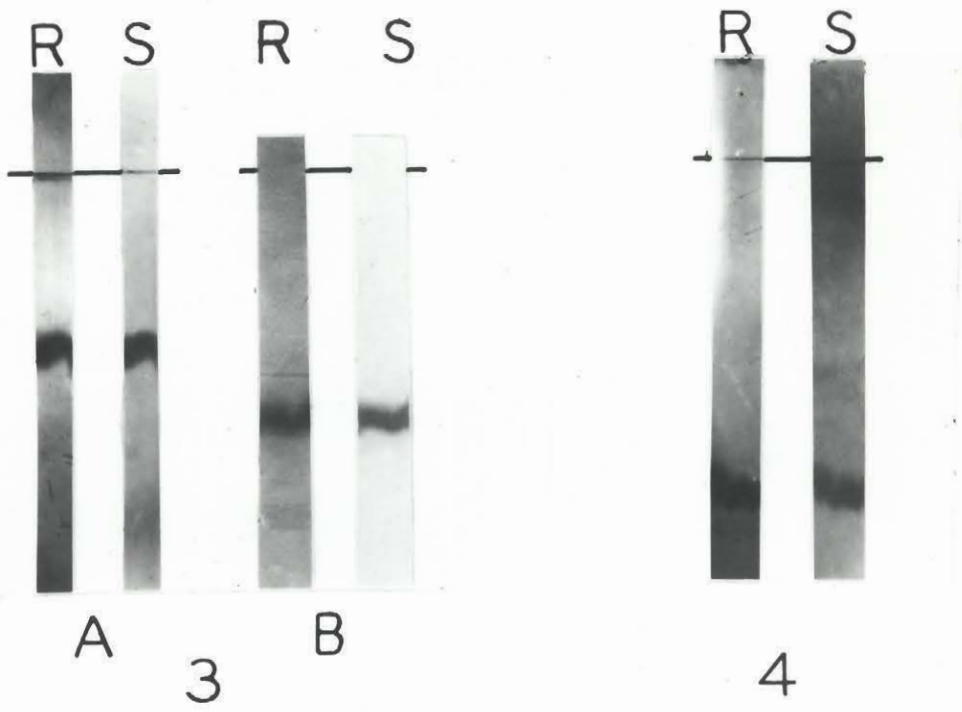



FIGURE 7A continued.

3A.R. Radioautogram of chromatogram of presumptive 16-oxooestradiol-17β zone after admixture with 25 micrograms of reference 16-oxooestradiol-17β and chromatography in system B.

3A.S. DSA stain of chromatogram.

3B.R. Radioautogram of similar mixed chromatogram after chromatography in system A.

3B.S. DSA stain of chromatogram.

4R. Radioautogram of chromatogram of presumptive oestradiol-17β zone after admixture with 25 micrograms of reference oestradiol-17β and chromatography in system B.

4S. DSA stain of chromatogram.

Note:- It was not possible to obtain satisfactory photographic reproductions for the presumptive oestradiol-17β zone after chromatography in system A although congruence of stain and radioautographic blackening was obtained.

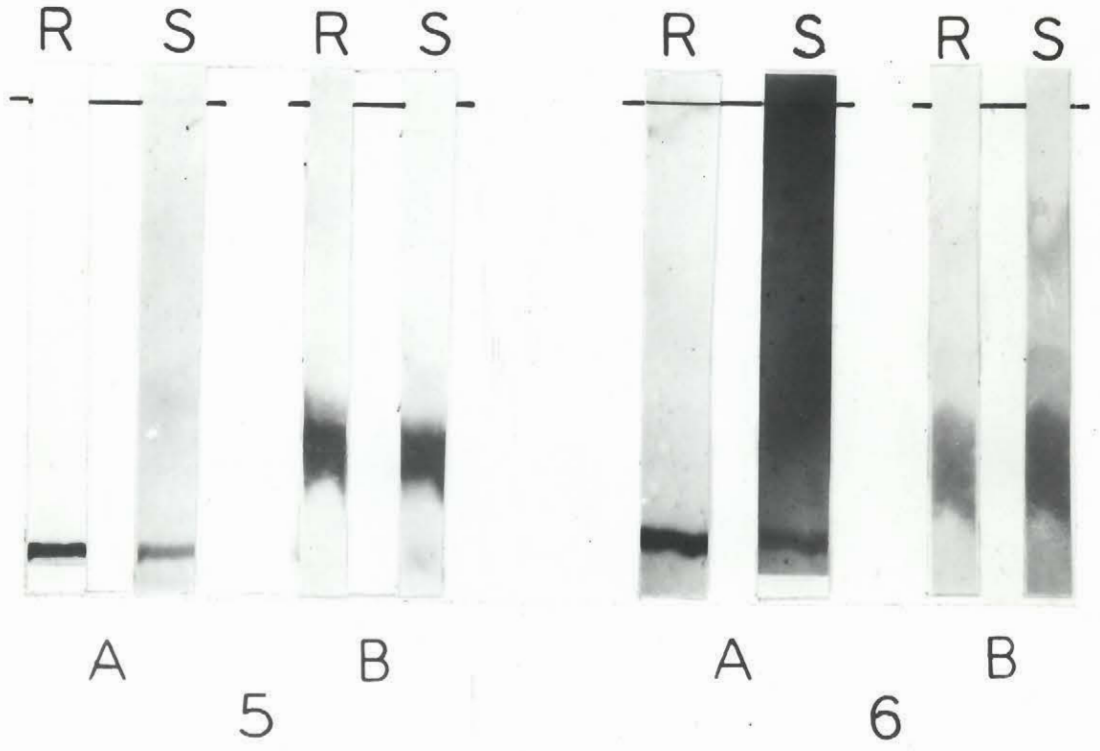



FIGURE 7A continued.

5A.R. Radioautogram of chromatogram of presumptive oxooestrone zone after admixture with 25 micrograms of reference 16-oxooestrone and chromatography in system C.

5A.S. DSA stain of chromatogram.

5B.R. Radioautogram of similar mixed chromatogram after chromatography in system A.

5B.S. DSA stain of chromatogram.

6A.R. Radioautogram of chromatogram of presumptive oestrone zone after admixture with 25 micrograms of reference oestrone and chromatography in system B.

6A.S. DSA stain of chromatogram.

6B.R. Radioautogram of similar mixed chromatogram after chromatography in system A.

6B.S. DSA stain of chromatogram.

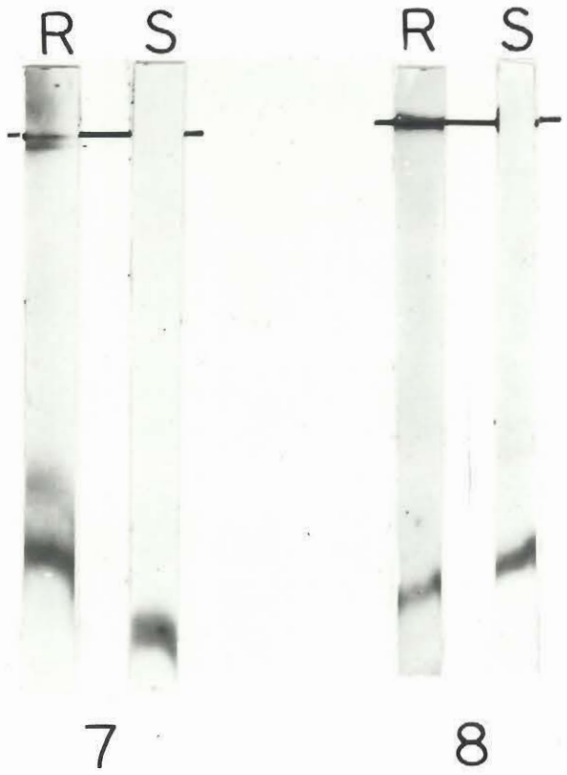



FIGURE 7(B) Attempted chromatographic identification of Zones 5 and 6A from the urinary extract after preliminary separation in system B.

7R. Radioautogram of chromatogram of suspected 16β-hydroxyoestrone zone (Zone 5) after admixture with 25 micrograms of reference 16β-hydroxyoestrone and chromatography in system B.

7S. DSA stain of chromatogram.

8R. Radioautogram of chromatogram of suspective 16α-hydroxyoestrone zone (Zone 6A) after admixture with 25 micrograms of reference 16α-hydroxyoestrone and chromatography in system B.

8S. DSA stain of chromatogram.

Note:- In neither case did the radioautographic blackening and the DSA stain display congruence as to shape and position. From this it was concluded that the Zones 5 and 6A are not identical with 16β-hydroxyoestrone and 16α-hydroxyoestrone respectively.

the laying hen presents a close parallel to the results obtained by Slaunwhite and Sandberg (1956) for the conversion of oestrone by a woman in the luteal phase of the menstrual cycle, in so far as both the laying hen and the non-pregnant woman excreted relatively large amounts of injected oestrone in the urine as oestrone and 16-oxooestrone.

Together with 16-oxooestrone, 17-epioestriol has been added to the list of conversion products of steroid oestrogens that have been detected in the urine of the laying hen. At the same time, the experiment failed to provide evidence for the presence of the hydroxyoestrones in the hen's urine, although it seems highly probable, on theoretical grounds, that they are present. No indication of the presence of ring B-unsaturated oestrogens or of 2-methoxyoestrogens has been obtained.

## 4. 2. EXPERIMENT 2.

### 4. 2. 1. Object.

This experiment was essentially a repition of Expt. 1, with the addition that measurements were made of (a) the recovery of injected radioactivity in urine and faeces (no account was taken of the bile or eggs); and of (b) the relative amounts of the various conversion products in the extracts of oestrogens from urine and faeces.

### 4. 2. 2. Method

Ten microcuries (0.223 mgm.) oestrone-16-$C^{14}$ in 2 ml. propylene glycol was injected into the wing vein of a Rhode Island Red laying hen furnished with exteriorized ureters. Urine and faeces were collected for two successive 24-hour periods and the oestrogen extracts prepared as outlined previously in Section 3.22. The oestrogen extracts from both urine and faeces were chromatographed once in System B, radioautographed and the radioactivity counted on the Forro strip counter as outlined in Section 3. 2. 7.

In order to obtain a count for the total radioactivity injected, 1 microcuric of oestrone-16-$C^{14}$ was taken and chromatographed in System B. The radioactivity was then counted on the Forro strip counter and the result multiplied by 10 to get the total count injected. The single peak on the tracing indicated the chromatographic purity of the oestrone.

4. 2. 3. Results and Discussion.

4. 2. 3. 1. Urinary extracts.

The results obtained after a single chromatographic run in System Bare presented in Fig. 8 and Fig. 9 and in Table II. The four less 'polar' zones of the chromatogram of the extracts, viz., Zones 7, 8, 9 and 10, separated cleanly and corresponded closely in $R_F$ values with Zones 7, 8, 9 and 10 respectively as shown in Fig. 5. Zone 1, Fig. 8 corresponded with Zone 1, Fig. 5, i.e., with oestriol. Zone 2, Fig. 8, was relatively more prominent than was Zone 2 in Fig. 5, and this was probably related to the fact that the former represented a separation obtained by a single chromatographic run without any preliminary removal of equol or associated contaminants.

Zone 3, Fig. 8, corresponded to 16-epioestriol but it was poorly resolved from Zone 4 as compared with Fig. 5. Zone 6, Fig. 8, corresponded with Zone 6, Fig. 5 and hence with 16-oxooestradiol-17β. In order to confirm this identification of Zone 6, it was cut out, eluted with methanol, mixed with 50 micrograms of reference 16-oxooestradiol-17β, evaporated, taken up in ethanol and subjected to reduction with sodium borohydride as outlined in Section 3. 2. 9. The reduction products were chromatographed in System B. There was congruence of staining and radioautographic blackening for two well-defined zones. The major zone (more

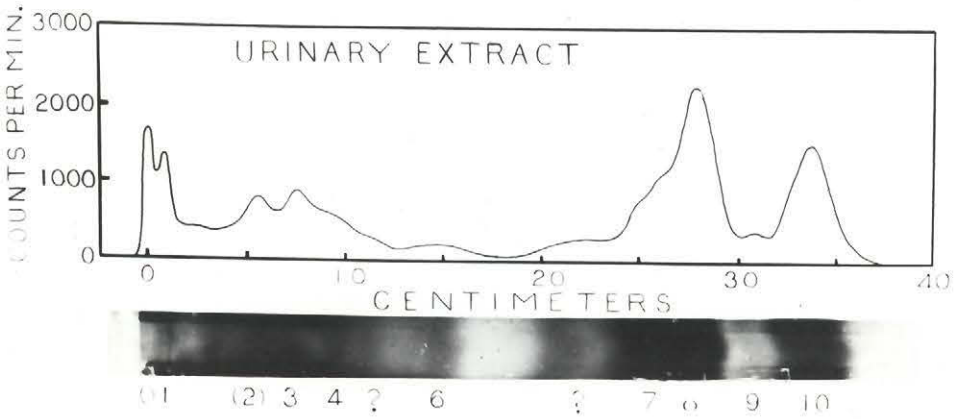



FIGURE 8. Radioautogram and plot of radioactivity of chromatogram (chloroform-formamide) of urinary oestrogen extract of laying hen after injection of oestrone-16-$C^{14}$. Enzymatic hydrolysis. Zones of urinary extract numbered as for Fig. 5. Zones which were neither identified, nor clearly correlated with any zones on Fig. 5 are marked with an interrogation mark.

Note: The chromatogram was run once only, to keep manipulative losses to a minimum.

FIGURE 9. Radioautogram of urinary oestrogen extract from second 24-hour period after injection of oestrone-16-$C^{14}$.

Note: Only two radioactive zones were detected. These corresponded to 16-oxooestrone (more 'polar' zone) and to oestrone (less 'polar' zone) on the basis of $R_F$ values with the corresponding zones in Fig. 5.

Radioactivities in different zones of chromatograms of urinary and faecal extracts in relation to radioactivity administered as oestrone-16-$C^{14}$ ($1.89 \times 10^5$ c.p.m. cm.).

Note:- (a) 24.5% of radioactivity injected was recovered in urinary and faecal extracts.

(b) 69% of the recovered radioactivity was in the urinary extract and 31% was in the faecal extract.

TABLE II

| | | 1st 24-Hours | | | 2nd 24-Hours | | CHROMATOGRAPHIC IDENTIFICATION |
|---|---|---|---|---|---|---|---|
| | Zone No. | Counts as measured. c.p.m. cms. | Counts as % of counts injected. | Counts as % of total urinary or faecal counts. | Counts as measured. c.p.m. cms. | Counts as % of counts injected. | |
| URINARY | 0 | 1822 | 0.96 | 6.1 | 283 | | Origin |
| | 1 | 1176 | 0.62 | 3.9 | | | Oestriol |
| | 2 | 1748 | 0.92 | 5.9 | | | 16-epioestriol and oestriol. |
| | 3 | 2142 | 1.13 | 7.2 | | | 16-epioestriol |
| | 4 | 1231 | 0.65 | 4.1 | | | 17-epioestriol |
| | 5(?) | 1340 | 0.71 | 4.5 | | | Unidentified |
| | 6 | 1659 | 0.88 | 5.6 | | | 16-oxooestradiol-17β |
| | 6A(?) | 1979 | 1.05 | 6.6 | | | Unidentified |
| | 7 | 2781 | 1.47 | 9.3 | | | Oestradiol-17β |
| | 8 | 6426 | 3.40 | 21.6 | 844 | | 16-oxooestrone |
| | 9 | 1074 | 0.57 | 3.6 | | | Unidentified |
| | 10 | 6426 | 3.40 | 21.6 | 1070 | | Oestrone |
| | Total | 29804 | 15.76 | 100.0 | 2197 | 1.16 | |
| FAECAL | 0 | 828 | 0.44 | 5.8 | ) | | Origin |
| | A | 442 | 0.23 | 3.1 | ) | | Oestriol (?) |
| | B | 2130 | 1.13 | 14.8 | ) | | 16-epioestriol |
| | ? | 1099 | 0.58 | 7.7 | ) Negligible | | Unidentified(unresolved |
| | C | 8534 | 4.52 | 59.5 | ) | | Oestradiol-17β |
| | D | 1312 | 0.69 | 9.1 | ) | | Oestrone |
| URINARY & FAECAL | Total | 14345 | 23.35 | - | 2197 | 1.16 | |

'polar') had an $R_F$ corresponding to 16-epioestriol. It was surmised that the second minor zone might correspond with 17-epioestriol, although Huffman and Lott (1955) have reported that borohydride reduction of 16-oxooestradiol-17β gives an almost quantitative yield of 16-epioestriol. There was one distinctive radioactive zone between Zones 6 and 7, but this was not identified.

The urinary extracts for the second 24-hour period (see Fig. 9) afforded only two distinct radioactive zones, which corresponded with Zones 8 and 10 respectively, as shown in Fig. 5. In order to get sufficient blackening on the X-ray film, it was found necessary to expose the chromatogram on X-ray film for two months.

4. 2. 3. 2. Faecal extracts.

The radioautogram of the faecal extract for the first 24-hour period showed resolution of four distinct radioactive zones in addition to the zone of application. The results are presented in Fig. 10 and in Table II. The radioautogram of the second 24-hour extract revealed no radioactive zones even after exposure of the chromatogram on X-ray film for 10 weeks.

Zone A had a mobility corresponding with that of oestriol. A mixed chromatogram of this zone with reference oestriol, when run in System A, did not give a clear-cut

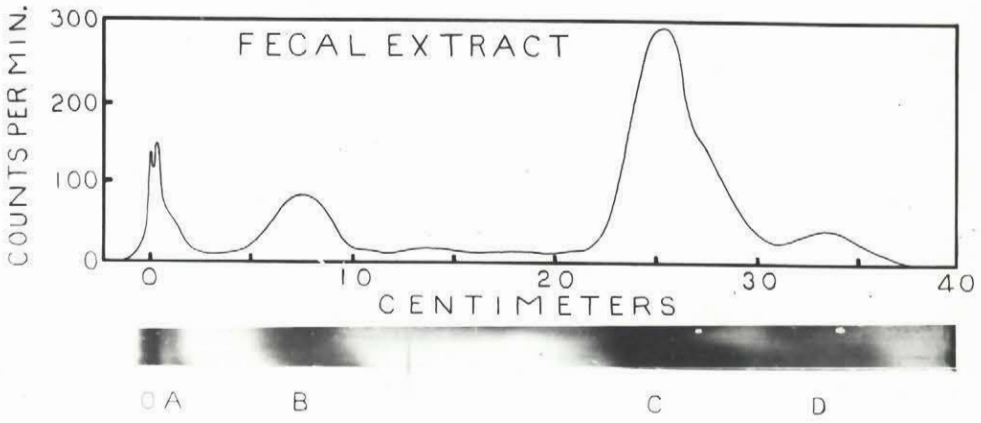



FIGURE 10. Radioautogram and plot of radioactivity of chromatogram (chloroform-formamide) of faecal oestrogen extract of laying hen after injection of oestrone-16-$C^{14}$. Enzymatic hydrolysis.

Zones indicated as follows:-

O. Zone of application.
B. Presumptive 16-epioestriol.
C. Presumptive oestradiol-17β.
D. Presumptive oestrone.

Note:- The chromatogram was run once only, in order to keep manipulative losses to a minimum.

congruence of stain and radioautographic blackening; nevertheless, it was thought likely that Zone A was oestriol.

Zone B gave good congruence of stain and radioautographic blackening when subjected to mixed chromatography with reference 16-epioestriol in System A.

Zone C was identified as oestradiol-17β and Zone D as oestrone by appropriate mixed chromatograms in System A.

The radioautograms of Zones B and C, together with the corresponding DSA-stained chromatograms are shown in Fig. 11. Although Zone D showed congruence of stain and radioautograms, the radioautographic blackening was insufficient to permit photography of the latter.

4. 2. 3. 3. Excretion of radioactivity.

The excretion of radioactivity is presented in Table II and shows that the recovery of injected radioactivity in the excreter was low, and only accounted for approximately one quarter of the total radioactivity injected. This low recovery does not appear to be a consequence of undue shortness of the collection period, for 23.4 percent of the radioactivity injected was recovered from the excreta in the first 24-hour period and only 1.16 percent from the excreta in the second 24-hour period. Some of the radioactivity may have passed to

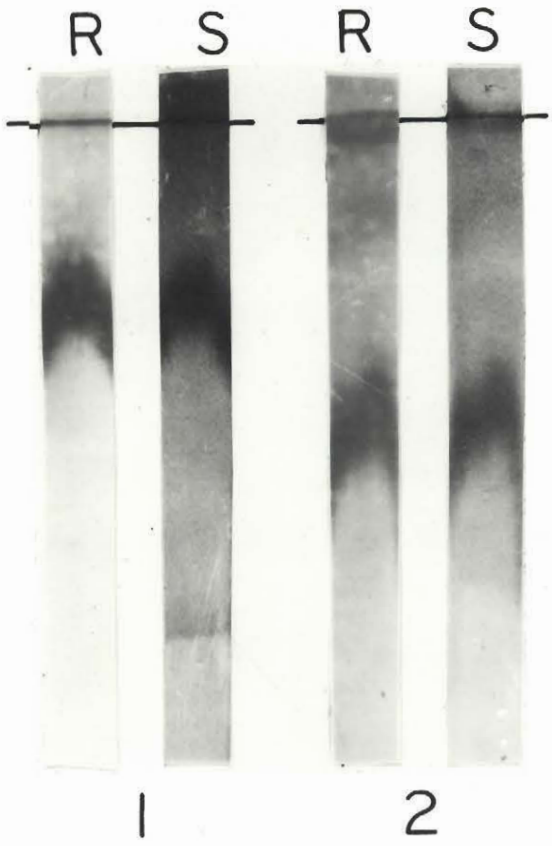



FIGURE 11. Chromatographic identification of presumptive 16-epioestriol (1) and oestradiol-17β (2) from faecal extract of laying hen after injection of oestrone-16-$C^{14}$, and preliminary separation in system B (see Fig. 10).

1R. Radioautogram of chromatogram of presumptive 16-epi-oestriol zone after admixture with 25 micrograms of reference 16-epioestriol and chromatography in system A.

1S. DSA stain of chromatogram.

2R. Radioautogram of chromatogram of presumptive oestradiol-17β zone after admixture with 25 micrograms of reference oestradiol-17β and chromatography in system A.

2S. DSA stain of chromatogram.

Note: The other two faecal zones were identified by similar means, but could not be reproduced satisfactorily by photography (see text).

the eggs, but this point was not studied. Part of the loss may have been due to absorption of radiation by contaminants on the chromatograms as well as to manipulative losses. Moreover, no account was taken of the spent urine after extraction with ether, or of the possibility that some of the radioactivity may have been extracted in the acidic fraction.

Of the individual fractions on the urinary chromatogram, the biggest recoveries were realized for the oestrone and the 16-oxooestrone zones. Each of these zones contained approximately one-fifth of the total radioactivity on the chromatogram, and more than twice as much as did the 16-epioestriol or oestradiol zones.

The faeces contained approximately one-third of the radioactivity recovered from the entire excreta. The observation supports the view that the faecal route is quantitatively important for oestrogen excretion in the fowl; quantitative studies of oestrogen excretion in the fowl must take account of this circumstance.

Zone C (oestradiol-17β) accounted for approximately 60 percent of the radioactivity on the chromatogram of the faecal extract, Zone B (16-epioestriol) accounted for approximately 15 percent, and Zone D (oestrone) accounted for approximately 9 percent.

A noteworthy feature of the radioautograms and the scanning patterns was the complete absence of any indication of the presence of metabolites less 'polar' in System B than oestrone. This suggests that 2-methoxyoestrone was absent, and this in turn suggests that 2-methoxy- or 2-hydroxyoestrogens may not be significant as urinary metabolites of oestrone in the laying hen.

It is of interest to compare the foregoing results, with those obtained by Beer and Gallagher (1955a, 1955b). These workers showed that the pattern of excretion of radioactivity was similar after either a large or small dose (administered intravenously or intra muscularly) of oestradiol-17β-16-$C^{14}$ or oestrone-16-$C^{14}$. They were able to account for approximately 65% of the injected radioactivity within four days following injection. It was also shown that the faeces were almost devoid of radioactivity on the first day after injection. The excretion of radioactivity in the faeces reached a peak on the second day, and by the seventh day, the faecal excretion of radioactivity equalled the urinary excretion. However, the faecal excretion following either a small or large dose was small (10%) as compared with the urinary excretion (55%). In the present experiment only 24.5% of the radioactivity was excreted within 48 hours and of this 31 percent was excreted in the faeces. At the present time

no explanation can be offered as to why the recovery of radioactivity is so low. At the same time, however, it may be possible that the fowl may be specially active in metabolizing oestrogens to non-phenolic metabolites or to unstable or difficultly extractable metabolites, such as 6-hydroxy-oestrogens. Since no account was taken of the possible presence of radioactivity in the acidic fraction or in the spent urine, this hypothesis remains to be tested.

A similar study on the recovery of injected oestrone-16-$C^{14}$ or oestradiol-17β-16-$C^{14}$ has been made by Sandberg and Slaunwhite (1957). Some of the experimental subjects had bile-fistulas (T-tube drainage). A little over 80 percent of the administered radioactivity was recovered in the urine during the 96 to 120 hours following the injection of these oestrogens. Approximately 50 percent appeared in the bile of the patients with T-tube drainage and about 7 percent appeared in the faeces of non-fistula patients, indicating gastrointestinal reabsorption of metabolites excreted in the bile. On the basis of these results, these authors postulated an hepato-biliary-enteric circulation of the injected oestrogen, in which about one-third to one-half of the administered oestrogen appeared in the bile. This, they postulated is deconjugated in the gastrointestinal tract and then reabsorbed. Following reabsorption some of the metabolites

are conjugated again in the liver and excreted in the urine, but the preponderant part is reconjugated and re-excreted in the bile to repeat the cycle just described. This continuous recirculation of metabolites accounts for the slow and delayed excretion in the urine of the radioactivity following the administration of oestrone-16-$C^{14}$ or oestradiol-16-$C^{14}$. This hepato-biliary-enteric circulation has been accepted in the past to be true in the cow (Longwell and McKee, 1942) and in the dog (Cantarrow et al, 1943; Pearlman et al, 1948) and it may be that it exerts a considerable influence on the excretion of injected oestrogen in the fowl.

All these suggestions are entirely speculative in the case of the laying hen. However, it seems likely that the low recovery of the injected radioactivity may in part be accounted for by considering the above statements.

## 4. 3. EXPERIMENT 3.

### 4. 3. 1. Object.

It is well-known that acid hydrolysis leads to some destruction of the 'classical' oestrogens and to complete destruction of the 16-hydroxyoestrones and of 16-oxooestrone. 16-Oxooestradiol is also largely destroyed by acid hydrolysis. Accordingly, enzymic hydrolysis of urine must be employed if these compounds are to be detected or isolated.

Experiment 3 was designed primarily to study the acid-lability of the various zones distinguishable on radio-autograms of the oestrogens from enzymatic hydrolysates of laying hen's urine.

### 4. 3. 2. Method.

Ten microcuries oestrone-16-$C^{14}$ were injected into the wing vein of a Rhode Island Red laying hen furnished with exteriorized ureters. Urine and faeces were collected for the next two succeeding 24-hour periods.

The urine for the first 24-hour period was diluted to 1 litre and the diluted urine was divided into two equal portions The first portion was subjected to enzymatic hydrolysis and worked up for oestrogens as described previously in Section 3.2.2. The second portion was subjected to acid hydrolysis by adding 50 ml. conc. HCl and refluxing the mixture for one hour. The oestrogen fraction was extracted by

Bauld's procedure as outlined by Bauld and Greenway (1957), but with omission of the separation into hydrophilic and lipophilic fractions.

The methanolic extracts from each portion were chromatographed in System B. The chromatograms were run until the solvent front had almost reached the end of the strip. The papers were then removed from the chromatography chamber, allowed to air-dry, and counted on the strip counter in order to compare the recoveries of injected radioactivity with the recoveries in Experiment 2.

Since the resolution of the zones was no better than that shown on Fig. 8, the papers were eluted with methanol and rechromatographed in System B. In this second chromatography the papers were allowed to air-dry after the first run in the solvent system and then redeveloped as before in the same solvent system.

4. 3. 3. Results and Discussion.

Pasieka et al (1956) and Pasieka (1960) have found this technique of redevelopment to be advantageous in the resolution of amino acids on chromatograms. In the author's experience redevelopment improved greatly the separation of radioactive zones on chromatograms in System B, although it naturally alters the $R_F$ values.

After the first chromatographic run in System B, the total radioactivity recovered after enzymatic hydrolysis was equivalent to 15,600 c.p.m. cms. and after acidic hydrolysis it was equivalent to 10,600 c.p.m. cms. Since only one-half of the urine was subjected to either enzymatic or acidic hydrolysis, the total counts obtained were doubled for purposes of comparison with the results obtained in Experiment 2.

The elution from the first chromatogram and rechromatography resulted in a loss of 49.7 percent and of 54.5 percent of the radioactivity on the original enzymatic and acidic hydrolysates respectively. The resolutions of the radioactive zones are shown by radioautogram and scanning patterns in Fig. 12. The quantitative data for the partition of radioactivity between the zones are presented in Table III.

A comparison of Fig. 12 with Fig. 8 shows that technique of redevelopment largely overcame the artificial separation of part of the 16-epioestriol in Zone 2 and this despite the fact that equol had not been removed before chromatography. The resolution of Zones 3 and 4 was sharpened by redevelopment.

It will be noted that there are minor discrepancies between the number of zones distinguishable on the radioautograms and the peaks distinguished on the scanning patterns over the region between Zones 6 and 7, but that otherwise

FIGURE 12. Radioautograms and plots of radioactivity of chromatograms (chloroform-formamide) of urinary oestrogens of laying hen obtained after (i) enzymatic hydrolysis and after (ii) acidic hydrolysis.
Zones numbered as in Fig. 5.

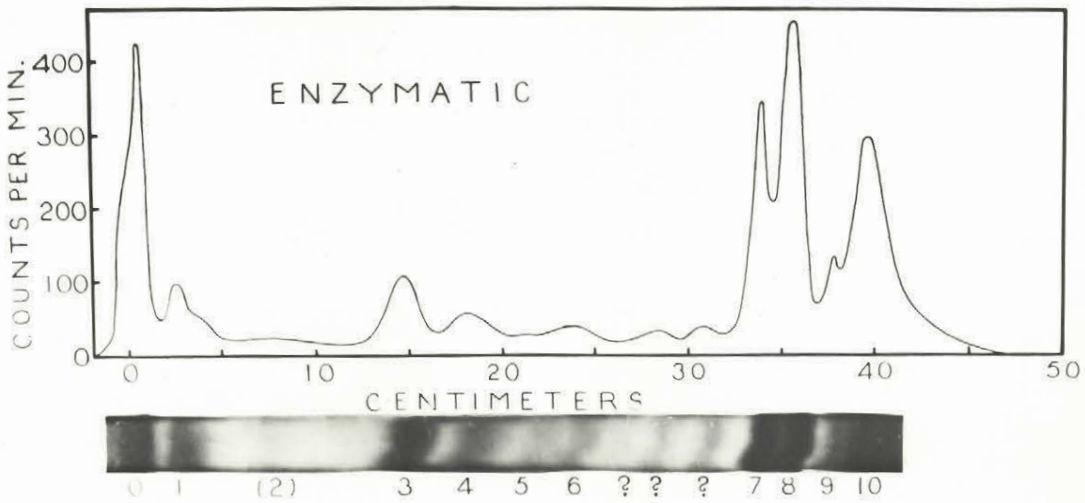
Note: (1) Effect of redevelopment technique (see text) in obviating Zone 2.

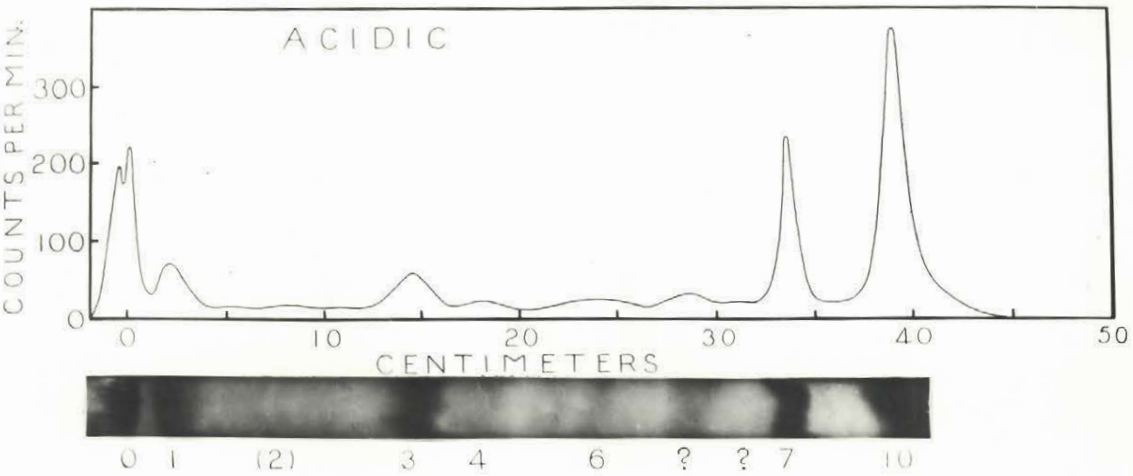
(2) Acid lability of material of Zones 8 and 9.

(3) Presence of unidentified zones on both chromatograms between Zones 6 and 7.

(4) Absence of any indication of metabolites less 'polar' than oestrone.

The radioautograms in this figure are shorter than the scanning patterns because the chromatograms were slightly too long to permit of radioautography on a single sheet of X-ray film.


ENZYMATIC
COUNTS PER MIN.
400
300
200
100
0
0
10
20
30
40
50
CENTIMETERS
0 1 (2) 3 4 5 6 ? ? ? 7 8 9 10


ACIDIC
COUNTS PER MIN.
300
200
100
0
0
10
20
30
40
50
CENTIMETERS
0 1 (2) 3 4 6 ? ? 7 10

Relative yields of urinary metabolites of oestrone-16-$C^{14}$ on chromatograms (a) after enzymatic hydrolysis and (b) after acidic hydrolysis. Results for first 24-hours after administration of oestrone-16-$C^{14}$.

✗ Although this zone was distinguished on the radioautogram, it did not give a recognizable scanning peak and the counts concerned were, therefore, divided equally between the two flanking zones, i.e. in accordance with the scanning pattern rather than the radioautogram.

## TABLE III

| Zone No. | ENZYMATIC HYDROLYSIS: Counts as measured. c.p.m. cms. | ENZYMATIC HYDROLYSIS: Counts as % of total urinary. | ENZYMATIC HYDROLYSIS: $R_F$ | ACIDIC HYDROLYSIS: Counts as measured. c.p.m. cms. | ACIDIC HYDROLYSIS: Counts as % of total urinary. | ACIDIC HYDROLYSIS: $R_F$ | Chromatographic Identification |
|---|---|---|---|---|---|---|---|
| 0 | 2756 | 16.0 | 0 | 1584 | 16.1 | 0 | Origin |
| 1 | 1100 | 6.4 | 0.06 | 718 | 7.3 | 0.06 | Oestriol |
| (2)3 | 1254 | 7.3 | 0.31 | 842 | 8.6 | 0.31 | 16-epioestriol |
| 4 | 794 | 4.6 | 0.38 | 350 | 3.6 | 0.39 | 17-epioestriol |
| 5 | 242 | 1.4 | 0.46 | - | - | 0.46 | Unidentified |
| 6 | 732 | 4.2 | 0.52 | 608 | 6.2 | 0.52 | 16-oxooestradiol-17β |
| ?× | - | - | 0.56 | - | - | 0.63 | Unidentified |
| ? | 366 | 2.1 | 0.61 | 510 | 5.2 | - | Unidentified |
| ? | 392 | 2.3 | 0.67 | 222 | 2.3 | 0.67 | Unidentified |
| 7 | 1950 | 11.3 | 0.73 | 1502 | 15.3 | 0.73 | Oestradiol-17β |
| 8 | 3158 | 18.3 | 0.77 | - | - | - | 16-oxooestrone |
| 9 | 608 | 3.5 | 0.81 | - | - | - | Unidentified |
| 10 | 3900 | 22.6 | 0.86 | 3508 | 35.6 | 0.86 | Oestrone |
| Total | 17252 | 100.0 | | 9844 | 100.2 | | |

there was excellent agreement between radioautogram and scanning pattern.

The results demonstrated that the material of Zone 8 was completely, or almost completely, destroyed in the course of acid hydrolysis. This observation provided further confirmation of the identification of Zone 8 as 16-oxooestrone. A very faint zone between Zones 7 and 10 on the radioautogram of the acidic hydrolysate may or may not represent a minute residue of 16-oxooestrone which had survived acidic hydrolysis.

Neither the radioautograms nor the scanning patterns provided any evidence that Zone 7 contained any radioactive metabolite other than oestradiol-17β. Zone 7 was cut out of the chromatogram of the acidic hydrolysate and run in System D as a mixed chromatogram with reference oestradiol-17β. There was congruence of staining with DSA and radioautogram of the single zone, and this gave further support to the view that oestradiol-17α is probably not a metabolite of oestrone in the fowl.

Comparisons of the two radioautograms in Fig. 12 and of their scanning patterns show that the material of Zone 9 was largely destroyed in the course of acid hydrolysis. A very faint zone on the radioautogram of the acidic hydrolysate in the position of Zone 9 may or may not have corresponded with a slight residue of the material of Zone 9 as seen on

the radioautogram of the enzymatic hydrolysate.

Zone 5 was only partly destroyed by acidic hydrolysis, an observation that supported the non-identity of Zone 5 with 16β-hydroxyoestrone.

Zone 6, identified by mixed chromatogram as 16-oxo-oestradiol-17β, was weakened but not destroyed by acidic hydrolysis.

The enzymatic hydrolysate showed the presence of two distinct radioactive zones on its radioautogram and of two distinct corresponding peaks on its scanning pattern in the region between Zones 6 and 7. The radioautogram also showed a third faint more 'polar' zone in this region. None of these zones has as yet been identified.

Both the radioautogram and the scanning pattern of the acidic hydrolysate showed, in the region between Zones 6 and 7 one major zone and a second weaker zone. The exact correspondences between these two zones and the three unidentified zones of the enzymatic hydrolysate calls for further study.

The quantitative data for the relative yields, as ascertained from the final scanning patterns, are presented in Table IV. The distribution of the radioactivity on the 'enzymatic' chromatogram was in good general agreement with

TABLE IV

| | Experiment 3. | | Experiment 2[xxx] |
|---|---|---|---|
| | Enzymatic hydrolysis | Acidic hydrolysis. | Enzymatic hydrolysis. |
| Total in urine. | 31,200[x] | 21,200 | 29,800 |
| as 16-oxooestrone | 5,710[xx] | nil | 6,426 |
| as zone 9. | 1,090[xx] | nil | 1,074 |
| as 16-oxooestrone plus Zone 9. | 6,800 | nil | 7,500 |

Comparison of recoveries of radioactivity in urine of laying hen (a) after enzymatic hydrolysis and (b) after acidic hydrolysis. Results expressed as counts per min. cm. per first 24-hours urine after administration.

[x] Counted from first chromatographic run.

[xx] Calculated on basis of distribution after second chromatographic run (Table III).

[xxx] From Table II.

that obtained in Experiment 2 as set out in Table II.

The magnitude of the difference between the total counts per 24-hour urine after enzymatic hydrolysis and after acidic hydrolysis is summarized in Table IV. It may be noted that the greater part of the difference could be accounted for by the destruction of 16-oxooestrone and of Zone 9 in the course of acidic hydrolysis.

The urinary chromatograms in this experiment provided an opportunity to measure the relative amounts of radioactive 16-epioestriol and radioactive 17-epioestriol, both zones having been identified by mixed chromatograms in System A subsequent to scanning. The data are summarized in Table V.

In both chromatograms the 16-epioestriol zone contained more radioactivity than did either the oestriol zone or the 17-epioestriol zone. The ratio of oestriol to 16-epioestriol in the urine is approximately 1:1. This is in contrast to human urine, in which the ratio of oestriol to 16-epioestriol is approximately 20:1 (Givner et al, 1960b). These results confirm a previous suggestion (MacRae & Common, 1960) that 16-epioestriol is quantitatively important as an oestrogen metabolite in the fowl.

The ratio of 16-epioestriol to 17-epioestriol was

TABLE V

| | Total counts per min. cm. | |
|---|---|---|
| | Enzymatic hydrolysis. | Acidic hydrolysis. |
| Oestriol. | 1100 | 718 |
| 16-epioestriol | 1254 | 842 |
| 17-epioestriol | 794 | 350 |

Relative amounts of radioactive oestriol, 16-epioestriol and 17-epioestriol recovered from the urine of laying hen after injection of oestrone-16-$C^{14}$.

approximately 2:1. In this connection it is of interest that MacRae, Dale and Common (1960) failed to observe 17-epioestriol in the urinary extracts following the administration of oestriol-16-$C^{14}$ to a laying hen. These observations would accord with the view that urinary 17-epioestriol is formed from oestrone in the fowl via 16α-hydroxyoestrone, but not via 16-oxooestradiol-17β.

## 5. SUMMARY

1(a). Oestrone-16-$C^{14}$ was administered intravenously to a laying hen furnished with exteriorized ureters. Urine was collected for 24 hours following injection and examined for oestrogens by a method involving enzymatic hydrolysis.

Radioautography of the resulting chromatogram yielded twelve radioactive zones. Of these zones, seven have been identified by chromatography of the appropriate radioactive zone with the appropriate reference material in two solvent systems.

By this means the following conversion products of oestrone were tentatively identified; oestriol, 16-epioestriol, 17-epioestriol, 16-oxooestradiol-17β, oestradiol-17β and 16-oxooestrone. Oestrone was also identified in the urine.

1(b) Equol was identified on the chromatogram of the urinary extract in amounts sufficient to give a positive DSA stain. Evidence is advanced in support of the suggestion that Zone 2 is formed as a result of radioactivity being held back by the equol zone.

1(c) Three radioactive zones were not identified viz. Zones 5, 6A and 9. However, by appropriate mixed chromatography of these zones, the presence of 16α- and 16β-hydroxyoestrone and of the ring B unsaturated oestrogens, was

eliminated.

1(d) The results have added 16-oxooestrone and 17-epioestriol to the list of conversion products of steroid oestrogens that have been detected in the urine of the laying hen after injection of the appropriate radioactive precursor.

2(a) In a second study a quantitative examination was made of the urine and faeces of a laying hen for conversion products of injected oestrone-16-$C^{14}$. Urine and faeces were collected separately for two successive 24-hour periods and worked up for oestrogens by the same method as for Experiment 1. By measuring the radioactivity on the chromatograms of the urinary and faecal extracts, it was shown that 24.5 per cent of the injected radioactivity was excreted within 48 hours following injection of oestrone-16-$C^{14}$. Of the radioactivity thus excreted, 69 per cent was excreted *via* the urine and 31 per cent *via* the faeces.

2(b) The urinary radioautogram for the first 24-hour period displayed the same general pattern as in Experiment 1. Measurements of the radioactivity present in each individual zone suggested that oestrone and 16-oxooestrone were excreted in greater amounts than the other conversion products. In this experiment, no estimate was given for the ratio of

oestriol to 16-epioestriol because of the presence of Zone 2. 15.76 Per cent of the radioactivity injected was accounted for during this period.

The urinary radioautogram for the second 24-hour period showed the presence of 3 radioactive zones. One of these zones corresponded to the origin, the second zone to 16-oxo-oestrone and the least 'polar' zone to oestrone. Only 1.16 per cent of the radioactivity injected was excreted during this period.

2(c) The faecal extract for the first 24-hour period yielded six radioactive zones. Four of these zones were identidied as oestriol, 16-epioestriol, oestradiol-17β and oestrone by appropriate mixed chromatograms. 7.59 Per cent of the total radioactivity was excreted during this period. Almost 60 per cent of this radioactivity was accounted for by the oestradiol-17β zone. Radioactive conversion products could not be detected in the faecal extracts for the second 24-hour period.

2(d) The radioautograms and the scanning patterns of both the urinary and faecal extracts gave no indication of the presence of metabolites less 'polar' than oestrone in the chloroform-formamide system. This indicates the absence of 2-methoxyoestrone and this in turn suggests that 2-methoxy-

or 2-hydroxyoestrogens may not be significant as urinary metabolites of oestrone in the laying hen.

2(e). The possible reasons for the relatively low percentage recovery of injected radioactivity are discussed.

3(a) In a third experiment, oestrone-16-$C^{14}$ was injected into a laying hen, and the urine was collected during the succeeding 24-hour period. This urine was divided into two equal portions. The first portion was subjected to enzymatic hydrolysis and the second portion to acidic hydrolysis. After the first chromatographic run in the chloroform-formamide system, the total radioactivity recovered was equivalent to 15,600 c.p.m. cms. and after acidic hydrolysis it was equivalent to 10,600 c.p.m. cms. Further chromatography of the extracts after elution involved a modified technique in which the chromatogram was redeveloped after the first chromatographic run. This technique was found to increase considerably the resolution of the radioactive zones and also overcame the artificial formation of Zone 2, even though equol had not been removed prior to chromatography.

3(b) The results of this experiment showed that after acidic hydrolysis the material of Zone 8 was completely or almost completely destroyed. Furthermore, the radioautogram of the acidic hydrolysate indicated that the material of

Zone 9 was also virtually destroyed. The destruction of these two zones accounted for the greater part of the loss of radioactivity consequent on acidic hydrolysis. The distribution and amounts of radioactivity excreted in the urine during the first 24-hour period were in good agreement with those obtained in Experiment 2.

3(c) Measurement of the radioactivity in both the acidic and enzymatic hydrolysed urinary extracts showed that, in both cases, the 16-epioestriol zone contained more radioactivity than either the oestriol or 17-epioestriol zones, and that the oestriol zone in both cases contained more radioactivity than the 17-epioestriol zone. The ratio of oestriol to 16-epioestriol in the urine was found to be approximately 1:1 which is in great contrast to the ratio found in human urine(20:1). This result confirms a previous suggestion that 16-epioestriol is quantitatively important as a urinary metabolite in the fowl.

4. The results, considered as a whole, suggest that oestrone is converted, in the fowl, into products which are similar to those identified in human urine, with the possible exception that methoxylated oestrogens are formed in relatively minute amounts or, possibly, not at all.

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