ON THE NATURE AND DETERMINATION OF URINARY OESTROGENS OF THE DOMESTIC FOWL

A Thesis

by

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GENERAL INTRODUCTION

It is recognised that oestrogen action plays a central role in the physiology and biochemistry of avian reproduction. Exogenous oestrogen has been shown to bring about striking changes in immature pullets, both in the morphology of the secondary sex organs, particularly the oviduct, and in the composition of blood and other tissues; the more obvious changes taking place in the latter include the involvement of the bony skeleton in the metabolism and transport of the materials of the egg yolk and egg shell and the production of yolk material by the liver. These matters have been the subject of reviews by Nalbandov (1953), Lorenz (1954), Sturkie (1958), Urist (1959) and van Tienhoven (1959). The similarity of these changes to those which occur at puberty in the normal pullet clearly indicates the presence of endogenous oestrogen in the domestic fowl.

At present there is no satisfactory supplement to urinary assay for assessing the secretory activity of oestrogen-producing tissue in any species. In this respect, the domestic fowl presents a serious difficulty in that urine and faeces are voided together and can only be separated following surgical modification either by exteriorization of the ureters or exteriorization of the rectum. Furthermore, the importance of the faecal route for excretion of oestrogens in various species has been

increasingly recognised and considerable species differences exist with regard to the nature and amounts of urinary and faecal steroids, even as between mammals. It must be emphasised, therefore, that a qualitative investigation of the nature and interrelationships of the excreted steroids should be made prior to their estimation being undertaken; furthermore, care must be taken in applying to animal urines, which may contain unknown interfering constituents, methods designed for the analysis of human urine.

Investigations as to the nature of the gonadal hormones of the domestic fowl were first undertaken in this laboratory by Layne (1957). He succeeded in identifying progesterone in extracts of ovarian tissue from laying hens, and subsequently reported the chromatographic detection of oestradiol, oestrone and oestriol in extracts of ovaries from laying hens.

Work in the field was continued by MacRae (1960), who succeeded in identifying oestrone and oestriol in extracts of avian droppings. He was able also to isolate oestradiol-17β from the same source (MacRae, Zaharia and Common, 1959).

At this stage, work on the interconversions of oestrogens by the laying hen, using C^{14} labelled oestrogens, was initiated by MacRae (1960). He was able to demonstrate the presence of radioactive oestrone, oestradiol-17β, oestriol and 16-epioestriol in the excreta of a laying hen following

administration of oestradiol-17β-C^{14} (MacRae, Layne and Common, 1959; MacRae and Common, 1960). Radioactive oestradiol-17β, oestrone and oestriol were also detected in the bile and radioactive oestradiol-17β was detected in the blood. MacRae (1960) also furnished evidence for the presence of radioactive oestriol, 16-epioestriol and 16-oxooestradiol-17β in the urine of a laying hen following administration of oestriol-16-C^{14}. In the same experiment the faeces yielded radioactive oestriol, 16-epioestriol and 16-oxooestradiol-17β, the latter being present in comparatively small amounts. A fourth minor faecal radioactive product was not identified. Equol, which is believed to represent a metabolite of plant isoflavones, was also identified chromatographically in oestrogen extracts from both urine and faeces.

Experiments by the present author (Ainsworth, 1961) on the interconversions of oestrone-16-C^{14} in the domestic fowl gave chromatographic evidence for the presence of the following radioactive conversion products in the urine following administration of oestrone-16-C^{14}: oestriol, 16-epioestriol, 17-epioestriol, 16-oxooestradiol-17β, oestradiol-17β and 16-oxooestrone. Three other minor conversion products were not identified. 16-Oxooestradiol-17β and 16-oxooestrone were characterised more fully by chromatographic examination of their sodium borohydride reduction products; the latter compound was also almost completely destroyed if the urine was subjected to acidic instead of enzymic hydrolysis.

This thesis is concerned with: (a) the isolation and identification of the steroid oestrogens in the urine of the domestic fowl; (b) further studies on the interconversions of oestrogens in the domestic fowl using C^{14} labelled compounds; (c) studies on the applicability of Brown's (1955) method for the estimation of oestrogens excreted in the urine of the domestic fowl; and (d) the estimation of urinary oestrogen excretion in both laying and non-laying birds.

PART I

STUDIES ON THE NATURE OF OESTROGENS IN THE DOMESTIC FOWL AND THEIR INTERCONVERSIONS *IN VIVO*

CHAPTER I

REVIEW OF THE LITERATURE

A. STEROID OESTROGENS OCCURRING IN THE HUMAN SUBJECT.

The literature on oestrogens in the human organism has been, in recent years, the subject of some excellent and extensive reviews (Breuer, 1960a and 1962; Diczfalusy and Lauritzen, 1961; Adlercreutz, 1962). In this review, therefore, the author will present only a brief survey of the subject with particular reference to more recent developments.

1. ISOLATION.

Most of the oestrogens known to be present in the human organism have been isolated from pregnancy urine. It was from this source that Doisy *et al.* (1929) and Butenandt (1929) isolated and identified oestrone, and Marrian (1930a, 1930b) and Doisy *et al.* (1930) and Doisy and Thayer (1931) isolated oestriol. It was not until ten years later that Smith *et al.* (1939) and Huffman *et al.* (1940) succeeded in isolating and identifying oestradiol-17β from the urine of pregnant women.

These earlier studies involved great technical difficulties and no further oestrogens were detected until better methods had been perfected. The discovery of the

Kober reaction, which is highly specific for the natural oestrogens, was of major importance analytically in the discovery of the newer oestrogens isolated by Marrian's group in Edinburgh. Furthermore the development of radioactive tracer techniques has provided a means of identification of their metabolic products in extremely small amounts.

Since the isolation of 16-epioestriol from pregnancy urine by Marrian and Bauld (1954, 1955), the following oestrogens have been isolated and identified from human pregnancy urine: 16α-hydroxyoestrone (Marrian et al. 1957a, 1957b); 16β-hydroxyoestrone (Layne and Marrian, 1958a); 16-oxooestradiol-17β (Layne and Marrian, 1958b); 2-methoxy oestrone (Loke and Marrian, 1958); 18-hydroxy oestrone (Loke et al. 1958, 1959); 16, 17-epioestriol (Breuer and Pangels, 1959); and 17-epioestriol (Breuer, 1960b). Frandsen (1959) detected 2-methoxyoestradiol-17β in pregnancy urine, and identified the compound by countercurrent distribution studies of this substance and its derivatives in different solvent systems. Recently, Notchev and Stimmel (1962) have published a preliminary report on the isolation and identification of 2-hydroxyoestrone from pregnancy urine.

Using countercurrent distribution techniques, Engel et al. (1952) detected oestrone and oestriol in the urine of non-pregnant women. Since the development of chemical methods for estimation of urinary oestrone,

oestradiol-17β and oestriol (Brown, 1955; Bauld, 1956; Ittrich, 1958; Preedy and Aitken, 1961b), it has become possible to estimate the amounts of these substances not only in the urine of fertile women but also in the urine of postmenopausal women and men. Dingemanse et al. (1938) had already isolated and identified oestrone from the urine of men. Other oestrogen metabolites detected in non-pregnancy urine are: 16-oxooestrone (Serchi, 1953); 16-epioestriol (Watson and Marrian, 1956); 16α-hydroxyoestrone (Loraine, 1958); and oestranediol A and B (Marker et al. 1938). Nocke (1962) succeeded in isolating and identifying 16-epioestriol from non-pregnancy urine.

Radioactive 2-methoxyoestrone (Kraychy and Gallagher, 1957a, b; Engel et al. 1957), 2-methoxyoestriol (Fishman and Gallagher, 1958) and 2-hydroxyoestrone (Fishman et al. 1960b) have been isolated and identified as metabolites of oestradiol-17β-16-C^{14} in the urine of normal individuals. Axelrod et al. (1961) isolated and identified 2-hydroxyoestriol in the urine of women to whom 2-hydroxyoestradiol-17β had been administered. The detection of radioactive 16-oxooestrone in the urine following the administration of oestrone-16-C^{14} has also been reported by Slaunwhite and Sandberg (1956). Chang and Dao (1961, 1962) have reported the isolation and identification of radioactive 11β-hydroxyoestrone and 11β-hydroxyoestradiol-17β from the urine of a castrated and adrenalectomised woman with metastatic breast cancer

following the administration of a tracer dose of cortisone-4-C^{14} acetate. An interesting report by Salhanick and Berliner (1957) described the isolation and identification of equilenin in a feminizing adrenal carcinoma.

2. METABOLISM

The metabolic pathways for the oestrogens are very complex, a fact which is evidenced by the great number of different metabolites isolated from pregnancy urine.

The known metabolic transformations of the oestrogen molecule are hydroxylation, oxidation, reduction and methylation. Other possibilities are epimerization, epoxidation (Breuer and Knuppen, 1961; Knuppen and Breuer, 1962), and the formation of p-quinols (Hecker and Zayed, 1961a, 1961b; Hecker and Marks, 1963).

Excellent summaries of the known metabolic reactions which have been shown to occur both *in vivo* and *in vitro* have been presented by Adlercreutz (1962) and by Breuer (1962). The present state of knowledge concerning the intermediary metabolism of oestrogens may be summarized schematically as shown in Figure 1. According to Fishman *et al.* (1960a, 1961) oestradiol-17β is rapidly oxidized to oestrone, this substance serving as the precursor of oestriol. However, the demonstration of the presence of an oestradiol-

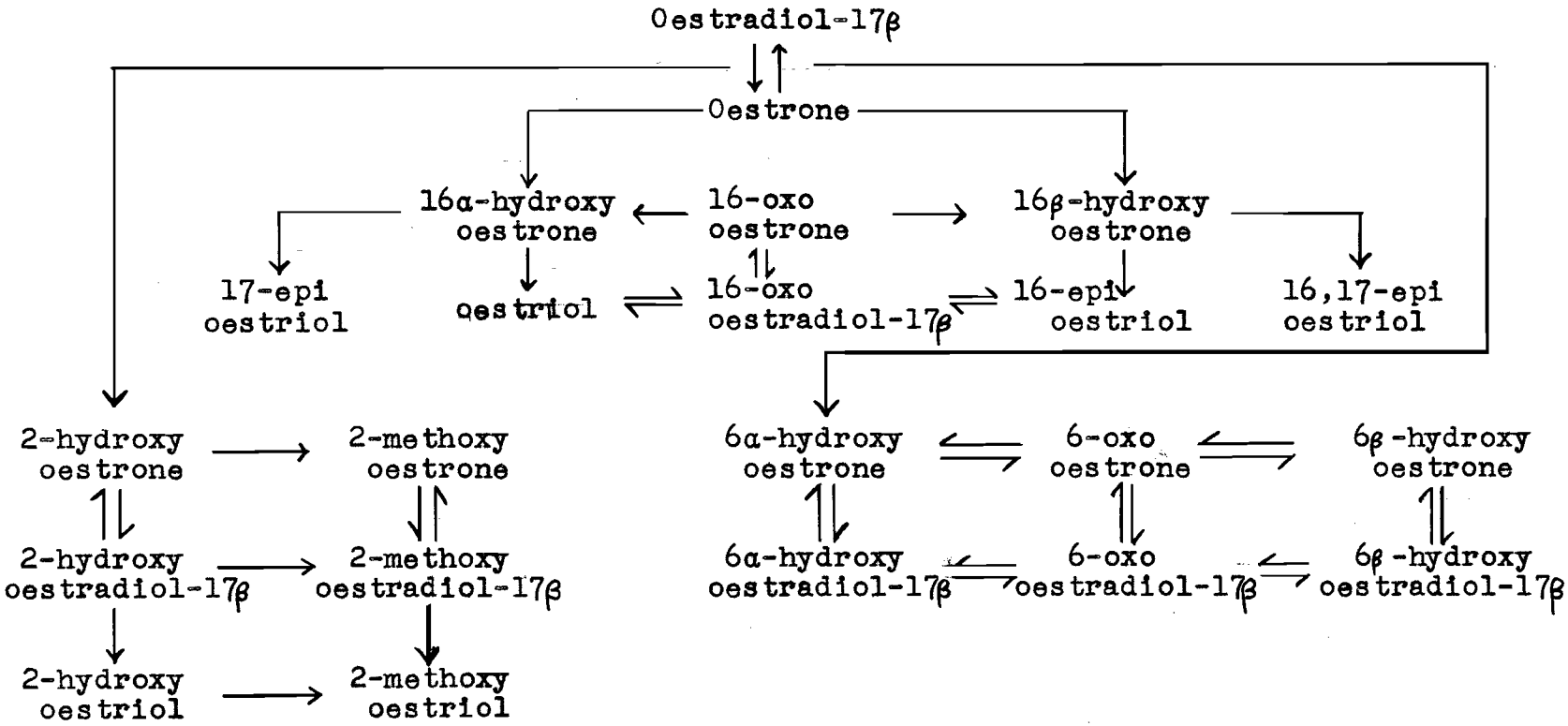


FIGURE 1. The metabolism of oestradiol-17β and oestrone in the human organism as evidenced by experiments *in vivo* and *in vitro* (from Adlercreutz, 1962).

17β : 16α hydroxylase in rat liver microsomes (Pangels and Breuer, 1962) suggests that oestradiol-17β may be a direct precursor of oestriol in the rat; and hence, it may be premature to abandon the classical view that oestradiol-17β can serve as an immediate precursor of oestriol in the human.

In a recent study, Hobkirk (1963) has shown that, following injection of oestrone-16-C^{14} into normal non-pregnant subjects, the α-ketol fraction subsequently obtained from the urine appears to consist of approximately equal amounts of 16α-hydroxyoestrone and a fraction containing 16-oxooestradiol-17β and perhaps 16β-hydroxyoestrone. Furthermore, the specific activities of the sodium borohydride reduction products of the α-ketol fraction were similar, indicating that the ketols arise from oestrone, perhaps via some common intermediate such as 16-oxooestrone (Breuer, Knuppen and Pangels, 1959).

Lucis and Hobkirk (1963) have made the interesting observation that human uterine fibroid tissue was capable of oxidizing 16-oxooestradiol-17β-16-C^{14} *in vitro* to 16-oxooestrone as well as reducing the former to material having characteristics of oestriol and 16-epioestriol. These findings indicate that this tissue is capable of carrying out reduction *in vitro* at C_{16} and also oxidation at C_{17} of the oestrogen molecule. Further observations indicated that human endometrium, Fallopian tube and fibroid

tissue are capable of reducing oestrone-16-C^{14} at C_{17} to form oestradiol-17β *in vitro*. However, the formation of oestrogen triols from labelled oestrone was not observed.

In a recent study, Breuer and Breuer (1963) have shown that following intramuscular injection of 16, 17-epioestriol into two men, 17-epioestriol and 16-epioestriol were isolated from the urine. They postulate that 17-epioestriol is formed from 16, 17-epioestriol via 16-oxooestradiol-17α, the latter then undergoing reduction to form 17-epioestriol. Further, the formation of 16-epioestriol must proceed via 16β-hydroxyoestrone, which is then reduced to form 16-epioestriol. The recoveries of 16, 17-epioestriol in the urine were 33% and 62% of the injected dose. These are lower than the 80% recovery found by Brown (1957) for oestriol. They conclude that 16, 17-epioestriol is metabolized to a greater extent than oestriol under comparable conditions.

B. STEROID OESTROGENS OCCURRING IN OTHER SPECIES.

1. ISOLATION.

All the isolations or identifications of oestrogen metabolites referred to above have been from human sources, but there are many reports of the occurrence of oestrogens in other animal species. The reported isolations of oestrogens from various domestic animals, including the horse, are listed in Table 1.

TABLE I

ISOLATION OF OESTROGENS FROM DOMESTIC ANIMALS

Species	Source	Oestrogen	Reference
Stallion	Urine	Oestrone	Hausler, 1934; Deulofeu & Ferrari, 1934.
		Oestradiol-17β Oestradiol-17α	Levin, 1945. Pigon et al. 1960, 1961.
	Testes	Oestrone	Beall, 1940.
Mare	Urine	Oestrone	De Jongh et al. 1931.
		Oestradiol-17β	Wintersteiner et al. 1935.
		Oestradiol-17α	Hirschmann and Wintersteiner, 1937-38.
		Equilin) Equilenin)	Girard et al. 1932 a, b, c.
		Dihydroequilin-17α) Dihydroequilenin-17α) Dihydroequilenin-17β)	Wintersteiner et al. 1936, Hirschmann and Wintersteiner, 1937-38, Glen et al. 1956, Gaudry and Glen, 1958.
		3-desoxyequilenin	Prelog and Fuhrer, 1945.
		$\Delta^{5,7,9}$-oestratrienol-3-one-17	Heard and Hoffman, 1940; Glen et al. 1958.
	Follicular fluid	Oestrone) Oestradiol-17β)	Short, 1960.
		6α-hydroxy oestradiol-17β	Bush, Klyne and Short, 1960.
Bull	Urine	Oestrone	Marker, 1939a.
Steer	Urine	Oestrone	Marker, 1939b.

TABLE I (continued)

Species	Source	Oestrogen	Reference
Cow	Urine	Oestrone Oestradiol-17α	Pope, McNaughton and Jones, 1957. Klyne and Wright, 1956a, 1959.
	Follicular fluid	Oestrone) Oestradiol-17β)	Short, 1962.
	Bile	Oestrone	Pearlman et al. 1947.
	Adrenal	Oestrone	Beall, 1939.
	Placenta	Oestrone) Oestradiol-17β) Oestradiol-17α)	Gorski and Erb, 1959.
	Meconium	Oestradiol-17α	Velle, 1957.
Goat	Urine	Oestrone) Oestradiol-17α)	Klyne and Wright, 1956b; 1957.
Sow	Urine	Oestrone	Velle, 1959.
	Ovary	Oestrone Oestradiol-17β	Westerfield et al. 1938. MacCorquodale et al. 1935, 1936.
Boar	Urine	Oestrone) Oestradiol-17β)	Velle, 1958d.

Although oestrone and oestradiol-17β have been found in all mammals studied so far, oestradiol-17α has been identified with certainty only in the urine of the pregnant mare, of the stallion, of the cow and of the goat; it has, however, also been isolated from the urine of rabbits after the administration of oestrone (Stroud, 1939) or of oestradiol-17β (Heard et al. 1941; Fish and Dorfman, 1941) and identified by paper chromatography in normal rat urine (Ketz et al. 1961). On this basis oestradiol-17α can be assumed to be a metabolic reduction product of oestrone rather than a primary secretory product. It has not been detected in human urine. It would seem, therefore, that as between the domestic animals, the rabbit and the rat on the one hand, and the human species on the other hand, there is a qualitative difference in the way oestrone is metabolically reduced. It should be noted also that the ring B unsaturated oestrogens in the urine of the pregnant mare (equilin, equilenin and the corresponding diols) have not been detected as normal excretory products in species other than the horse.

With regard to avian species, the only isolation so far, apart from the isolation of oestrone reported in the present thesis, would appear to be that of oestradiol-17β from avian droppings (MacRae et al. 1959). However, Hurst et al. (1957) have reported chromatographic evidence for the presence of oestrone and oestriol in the droppings of the male and female domestic fowl. In addition they

observed the presence of oestradiol in the droppings of the rooster. Layne et al. (1958) have also detected oestrone, oestradiol-17β and oestriol in the ovaries of laying hens.

The occurrence of oestriol in avian species is of 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 (Merrill, 1958). Dorfman (1957), however, has discussed the reported identifications of oestriol in species other than man and has stated that "these studies remove the myth that oestriol is an exclusive product of man."

A further point of interest is that the endocrine similarities between fish and higher vertebrates, which at one time were a matter of conjecture, are now becoming more evident.

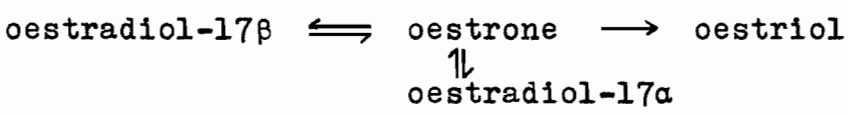
Oestrone and oestradiol-17β, the typical oestrogens of the ovary in higher vertebrates, have been identified in the ovaries of the ovoviparous elasmobranch fish Squalus suckleyi (Hisaw et al. 1960) and Squalus acanthias (Simpson et al. 1963a) and in the oviparous teleost Gadus callarias (Gottfried et al. 1962). A preliminary report by Chieffi (1962) that the ovaries of the ovovivaparous selachian Torpedo marmorata contain as the major oestrogens oestriol and oestradiol-17β should perhaps be treated with some

reserve, since the methods used have been the subject of criticism (Gottfried et al. 1962).

Oestrone and oestradiol-17β have been found also in the plasma of the elasmobranch S. caniculus (Simpson et al. 1963b). The same oestrogens have been found by Cedard and Normura (1961) in surprisingly large quantities in the blood of the Teleost fish Anguilla, Conger, Muraena and Cyprinus; oestriol could not be detected.

2. METABOLISM

A comparison of the chemical structures of the various oestrogens which have been obtained in exhaustive isolation studies (Table 1) suggest metabolic inter-relationships, some of which have been substantiated by experiment. It has been fairly well established that the following reactions occur in the mammalian organism:



This scheme was based originally on the isolation or detection of metabolites of the oestrogen under study following its administration in massive doses to an experimental subject. Since the literature on this aspect of the oestrogens has been summarised by Pearlman (1948) and by Dorfman (1955), a detailed report is not included here.

Whilst the metabolic transformation of oestrogens in the human species has been the subject of extensive

research, information on the metabolism of oestrogens in domestic animals is relatively scanty.

Levy *et al.* (1956) demonstrated the formation of oestradiol-17α on incubation of oestrone with bovine blood. Furthermore, Axelrod and Werthessen (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.

In a series of *in vivo* studies on the metabolism of oestrogens in the bovine, Velle (1958b, c, e) showed that the young calf was able to convert oestradiol-17β to oestrone and oestradiol-17α; oestradiol-17α to oestrone; and oestrone to oestradiol-17α. In similar experiments *in vitro* it has been shown that tissue cultures of cells from bovine kidney, testis, endometrium, amnion and liver, which had been grown on a medium free from blood, can all bring about the interconversion of oestrone and oestradiol-17β (Velle and Erichsen, 1960; Erichsen and Velle, 1960). However, only trace amounts of oestrone were found following incubations with oestradiol-17α in these tissues. Since the previous *in vivo* experiments had shown that the conversion of oestrone to oestradiol-17α takes place at a high rate (Velle, 1958c), it has been suggested that this reaction, which does not appear to take place *in vitro*, is mediated by an enzyme present in blood, since oestradiol-17α is the major metabolite following incubation of oestrone with bovine blood (Levy *et al.* 1956; Axelrod and Werthessen, 1960).

Portius and Repke (1960a, b) were able to show distinct differences in the steric specificity and activity of oestradiol dehydrogenases present in the erythrocytes from fifteen different species, the results being based on incubation experiments with oestradiol-17α, oestradiol-17β and oestrone. Their results showed that all the erythrocytes possessed a 17β-oestradiol dehydrogenase whilst only in the erythrocytes from cattle, sheep and goats was the corresponding 17α-dehydrogenase found. Furthermore, the β-enzyme was the most active in the rat and least active in man: and only in erythrocytes from cattle was the α-enzyme more active than the β-enzyme. In all cases, the enzymes were located exclusively in the red blood cells.

There appears to be very little information on the metabolism of oestrogens in the porcine species. However, an interesting experiment by Lunaas (1963) showed that there was a 40% net increase in the urinary oestrone fraction following intramuscular injection of oestradiol-17β. The oestradiol-17β recovered amounted to less than 2% of the dose. This is in contrast to the finding that the amount of oestradiol-17β in the ovaries apparently exceeds that of oestrone (Westerfield *et al.* 1938). These findings could be taken to indicate that in the sow oestradiol-17β, if released from the ovaries, is transformed peripherally prior to elimination rather than excreted as such. It is probable, also, that this efficient transformation observed *in vivo* takes place

outside the blood, since Portius and Repke (1960b) have shown that, in the presence of porcine erythrocytes, oestrone and oestradiol-17β will equilibrate at a ratio of about 1:2.

Further isolated experiments on oestrogen metabolism in mammals have shown that the mature male dog is capable of converting oestradiol-17β-6,7-H^3 into oestrone and oestradiol-17α (Siegel et al. 1962). Experiments by Trachewsky and Hobkirk (1963) have shown that 16-oxo-oestradiol-17β, when incubated with whole blood from the cat or the rat, was reduced to oestriol and 16-epioestriol: the former predominated in cat blood, the latter in rat blood. It is probable that these findings represent a species difference in the way the 16-oxo group is reduced.

The metabolism of oestrogens by the domestic fowl has also received some attention. MacRae et al. (1959) provided some evidence for the validity of the metabolic sequence, oestradiol-17β $\rightleftharpoons$ oestrone $\longrightarrow$ oestriol in the laying hen when they reported the detection of radioactive oestrone, oestradiol-17β and oestriol in extracts of bile and droppings of the laying hen, after intravenous injection of oestradiol-17β-16-C^{14}. Radioactive oestradiol-17β was also demonstrated in the blood. In a second study, MacRae and Common (1960) also demonstrated the presence of radioactive 16-epioestriol in the droppings following injection of the same precursor. Further studies by MacRae,

Dale and Common (1960) demonstrated the presence of radioactive 16-epioestriol and 16-oxooestradiol-17β in the urine of the laying hen following injection of oestriol-16-C^{14}. The results also showed the presence of appreciable amounts of radioactive 16-epioestriol in the faeces but little, if any, radioactive 16-oxooestradiol-17β.

In experiments on the conversion of intravenously administered oestrone-16-C^{14} by the laying hen, Ainsworth and Common (1962) were able to obtain chromatographic evidence for the presence of six radioactive conversion products in the urine excreted over the 24-hour period following injection. The radioactive conversion products detected were:- oestriol, 16-epioestriol, 17-epioestriol, 16-oxooestradiol-17β, oestradiol-17β and 16-oxooestrone. Further evidence as to the identity of the presumptive 16-oxooestradiol-17β and 16-oxooestrone was obtained by examination of their sodium borohydride reduction products (in which both yield 16-epioestriol as the predominant reduction product) and by the complete destruction of the latter following acidic hydrolysis of the urine samples. It was also shown that the principal radioactive material in the faeces was oestradiol-17β. Furthermore, 69% of the radioactivity recovered from the excreta was present in the urine.

<u>In vitro</u> studies by Mitchell and Hobkirk (1959) have shown that oestradiol-17β-16-C^{14} can be converted to radio-

active oestriol by avian liver slices.

The results of these radioactive tracer experiments with the domestic fowl, considered as a whole, suggest that the injected radioactive precursors are converted into products which bear a general similarity to those identified in human urine.

C. METHODS OF EXTRACTION AND PURIFICATION OF OESTROGENS.

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. However, the quantity of steroid present in most tissues and biological fluids is usually small (of the order of a few micrograms), and the amount of interfering material is frequently large. It is often necessary, therefore, that the methods employed should be capable of detecting and estimating amounts of the order of 5 to 100 micrograms of steroid or even less and rigorous purification is essential.

The present review is restricted to consideration of the extraction and identification of oestrogens from urine. For details on the extraction and identification of oestrogens from other biological materials, the reader is referred to reviews by Bauld and Greenway (1957), O'Donnell and Preedy (1961), Diczfalusy and Lauritzen (1961) and Preedy (1962), who have discussed at length all the important methods in the field.

1. HYDROLYSIS

Oestrogens are considered to be present in urine mainly in the form of glucosiduronates and ester sulphates. Since methods for the direct estimation of oestrogen conjugates are not currently available, it follows that hydrolysis is a necessary preliminary step in the identification of urinary oestrogens.

The classical procedure for the hydrolysis of oestrogen conjugates in urine is to reflux with 15% (v/v) hydrochloric acid for periods of 30 to 120 minutes. Various recommendations regarding the amount of acid to be added and the length of time of refluxing have been put forward. Bauld and Greenway (1957) have discussed the optimal conditions for hydrolysis of urinary oestrogens. Brown and Blair (1958) made a careful, detailed study of the optimal conditions for acid hydrolysis of urine. These authors investigated the maximal yield of oestrogens obtainable from urine under varying conditions, in terms of Brown's method for estimating urinary oestrogens (Brown, 1955). Maximal yields were obtained when 15-20 volumes of concentrated hydrochloric acid were added to 100 volumes of urine, and the solution was refluxed for 60 to 120 minutes. Within these limits the yield of oestrogen remained approximately constant. The authors were able to show that hydrolysis by this procedure was accompanied by some destruction of the "classical" oestrogens.

Enzymic hydrolysis has been used increasingly in the estimation of total oestrogens, particularly in urine. Material from bacterial sources and from organs of animals, shellfish and snails may contain large amounts of β-glucuronidases and/or sulphatases capable of hydrolysing oestrogen conjugates. The various enzyme preparations and their uses have been reviewed by Preedy (1962).

Enzymic hydrolysis offers several advantages over acid hydrolysis. Firstly, the treatment is much milder than acid hydrolysis and no significant destruction of oestrogens occurs (Brown and Blair, 1958). Secondly, acid hydrolysis tends to produce much interfering material, whereas this does not occur to the same extent with enzymic hydrolysis. Thirdly, enzymic hydrolysis is essential for the recovery of a number of the 'newer' oestrogens, including the ring D α-ketols, from urine, since these are unstable when refluxed with strong acid.

On the other hand, enzymic hydrolysis has the disadvantage of being expensive and time-consuming. Furthermore, it results generally in relatively incomplete cleavage of conjugates, due to the presence of inhibitors of both β-glucuronidase and sulphatase in human urine (Brown and Blair, 1958; Slaunwhite and Sandberg, 1960).

Bugge et al. (1961) compared the release of six oestrogen fractions from conjugation in human pregnancy

urine using enzyme preparations from mammalian liver (β-glucuronidase), from the snail Helix pomatia (β-glucuronidase + sulphatase), from the common limpet Patella vulgata (β-glucuronidase + sulphatase) and bacterial β-glucuronidase. The oestrogens concerned were, oestrone, oestradiol-17β, oestriol, 2-methoxyoestrone, 16-epioestriol and a ring D α-ketolic fraction (mainly 16α-hydroxyoestrone). The enzyme preparation from Helix pomatia proved to be superior to the β-glucuronidase enzymes of bacterial or hepatic origin in the case of oestrone and ring D α-ketolic oestrogens, considerable amounts of which may be conjugated in non-glucuronide form. Furthermore, the snail enzyme preparation gave higher yields than the corresponding limpet enzyme, suggesting a higher efficiency of hydrolysis. Very similar results were obtained for the ring D α-ketolic fraction, the snail enzyme preparation being the most efficient. However, marked decreases in the ring D α-ketolic and the oestrone fractions were observed between 12 and 96 hours when incubated with the snail enzyme preparation. Conventional hot acid hydrolysis, following dilution of the urine 1:10 with water, yielded levels of oestrone, oestradiol-17β and oestriol which agreed fairly well with those obtained following snail enzyme hydrolysis. Optimal hydrolytic conditions were realised by incubating for 24 hours with 500 units of β-glucuronidase and 250 units of sulphatase per ml. of urine at pH 5.2 and 37-38°C.

The use of ion exchange resins for separation and purification of oestrogen conjugates prior to hydrolysis has also been investigated. Bauld (1952) reported in brief on the use of the anion exchange resin (Amberlite IR4B) for the separation of oestrogen conjugates. Acid hydrolysis of the resultant solution of oestrogen conjugates eluted from the column gave no charring and a marked decrease in ether-soluble pigment formation. Quantitative elution, however, required large volumes of the eluting solution.

Recently, three methods for simultaneous purification and concentration of urinary conjugated oestrogens have been developed. Hahnel (1962) has described the quantitative removal of oestrogen conjugates from urine using the anion exchange resin De-acidite FF. The subsequent quantitative elution of the oestrogen conjugates from the resin column with a mixture of pH 6.7 buffer-ethanol yielded a solution which contained considerably less non-oestrogenic chromogens than the original urine. However, the method appears to be rather laborious, since large volumes of solution were required to elute the oestrogen conjugates quantitatively from the column. A more promising method is that which has been devised by Beling (1961, 1963). In this method the ion exchange column has been replaced by Sephadex gel (dextran crosslinked with epichlorhydrin) which differs in many respects from those procedures generally referred to as column chromatography. It seems that "filtration" most

adequately describes the operative mechanism. A great amount of experimental data has accumulated in the last few years (Porath, 1962) but no theory which can fully explain the mechanism of action of gel filtration has been presented. However, Porath (1962) has postulated a molecular sieve effect.

The application of gel filtration to the separation and purification of urinary conjugated oestrogens was presented in a preliminary report by Beling (1961). Samples of urine were applied directly to Sephadex G25 columns, and the conjugated oestrogens were eluted from the columns in two well defined peaks, using distilled water as eluent. In a further study, Beling (1963) has presented full details for the application of gel filtration to the purification and concentration of conjugated urinary oestrogens.

Kushinsky and Tang (1963a, b) have described methods whereby free and conjugated oestrogens may be extracted from urine. Their first procedure comprised the following steps: a 10 per cent (v/v) solution of a high molecular weight secondary amine (Amberlite LA-2) in ethyl acetate was washed with formic acid and water; the oestrogens in urine (acidified to pH 2 or 3 with sulphuric acid) were extracted with the LA-2 solution; the oestrogen fraction was then back-extracted from the organic solution with dilute aqueous ammonia. The extracts contained approximately 90

per cent of the oestrogens, were devoid of most of the extraneous material and could be reduced in volume to less than 1/15 of the original urine. Their second method consisted of coating Celite 545 with Amberlite LA-1 which was then used as a chromatographic support for the separation of free and conjugated oestrogens in urine and urine extracts (prepared by the LA-2, ethyl acetate procedure). Elution of the columns was carried out with extremely dilute acids. They found that optimal separation was obtained with a column of Celite coated with LA-1 (formate form) from which the conjugated oestrogens in a concentrated urinary extract were eluted with a linear gradient of formic acid (pH 3.10-5.04).

2. EXTRACTION

After hydrolysis of conjugates in the urine, the free oestrogens so released must undergo concentration and preliminary purification. 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. The preliminary purification depends on the design of a suitable extraction procedure which takes into account a consideration of oestrogen partition coefficients between various immiscible solvent pairs. The extraction procedures which have been generally applied to urine are those of Engel *et al.* (1950), of Brown (1955) and of Bauld

(1956). All three procedures depend upon the preliminary extraction of the urine with ether; a phenolic partition between sodium hydroxide and an organic solvent such as toluene, benzene or light petroleum, in which the oestrogens remain in the alkaline phase and neutral lipids enter the organic phase: and an ether extraction from an aqueous phase adjusted to pH 9.0-10.0.

The methods of Brown (1955) and of Bauld (1956) include in addition a partition between benzene-petroleum ether and water (1:1) in the case of the Brown method and a partition between benzene and water in the Bauld method. In this partition oestriol is quantitatively separated from oestrone and oestradiol-17β, the former being distributed in the aqueous phase and the latter two in the organic phase. The procedure of Engel et al. (1950) has the advantage that only one extraction sequence is involved, compared to two sequences in the methods of Brown (1955) and Bauld (1956). It must be emphasised that many variants of these procedures have been used. A summary of the findings which resulted in the development of these methods has been presented by Bauld and Greenway (1957).

In addition to the simple partition methods, several other methods have been used as additional purification steps prior to separation of the oestrogen fractions. Several chemical methods have been used for 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 derivative formation was applied in a most efficient form by Brown (1955). After separation of oestriol and 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 nonpolar 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. However, it has recently been shown by Hertelendy and Common (1963) that methylation of 16-epioestriol and of 17-epioestriol by Brown's procedure results in the formation of three methylation products of different chromatographic mobilities, thereby indicating that methylation cannot be applied as a purification step in the purification of these two cis-glycols for quantitative purposes.

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

Theoretically, ion exchange resins should be useful in the further purification of urinary extracts. Engel et al. (1961) described the use of Dowex 2 anion exchange resins, in the bicarbonate form, for the purification of radioactive urinary metabolites following the injection of oestradiol-17β-16-C^{14} into normal individuals. Total urinary extracts were dissolved in methanol and transferred to the previously prepared column. Methanol (70 ml.) was then passed through the column to elute the neutral fraction. The oestrogens were then eluted from the column with 5N acetic acid in methanol. The evolution of CO_2 was disregarded. By the use of this step a considerable purification, together with quantitative recovery of the oestrogen fraction from the column, as assayed by radioactivity measurements, was effected.

3. SEPARATION OF OESTROGENS FOLLOWING PRELIMINARY PURIFICATION.

The oestrogens and oestrogen metabolites which have been extracted and partially purified by the various procedures must then be separated and identified. It was at this stage that methodology was held up for many years through lack of appropriate techniques. The advent of chromatography made such separation possible for the first time. Several chromatographic techniques have been applied to the separation of oestrogens in biological fluids, particularly urine: paper chromatography; adsorption column chromatography; countercurrent distribution and column partition chromatography; and, more recently, gas chromatography and thin-layer chromatography.

Very useful reviews of the various chromatographic methods for separating oestrogens from biological materials have been presented by Bauld and Greenway (1957), O'Donnell and Preedy (1961) and Preedy (1962). An excellent review of the theoretical and practical aspects of chromatography as applied to steroids has been published by Bush (1961). More recently, Oakey (1962) has reviewed the application of paper chromatography to the separation of the oestrogens.

Two main types of filter paper partition chromatography have been used in the separation of steroid hormones. That introduced by Zaffaroni, Burton and Keutmann (1949) used paper impregnated with a high-boiling polar solvent,

usually propylene glycol or formamide, as the stationary phase. The mobile phase was 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.

The detection of steroids on paper can be 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. However, it must be emphasised 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 (1961) has tabulated appropriate methods of detection for many types of steroid material. Axelrod and Pulliam (1960) have devised six tests for the identification of oestrogens in micro quantities on filter paper and have tabulated the colours produced with each of these tests for eighteen oestrogenic compounds.

Adsorption column chromatography has been much used for the separation of oestrogens in body fluids and tissues. Alumina column chromatography is an essential part of the method of Brown (1955) for urinary oestrogens.

Silica gel columns have been used by Beer and Gallagher (1955a, b), Sandberg and Slaunwhite (1957) and Levitz et al. (1956) for the separation of urinary oestrogens.

Countercurrent distribution and column partition chromatography can be considered together, since they both depend upon the different partition coefficients of single substances between two immiscible solvent pairs. A description of countercurrent distribution has been given by Williamson and Craig (1947). Countercurrent distribution has been employed in the separation of urinary oestrogens by Engel et al. (1950, 1957), Gallagher et al. (1958), West et al. (1958a, b), and others.

In column partition chromatography, the stationary phase is adsorbed on to an inert powder such as kieselguhr (Celite) which acts as a support for the stationary phase and is called the supporting phase. The latter is then packed into a column along with the stationary phase, and a mobile phase is allowed to flow through the column. Under these conditions, Celite has virtually no adsorptive effect and the substances being chromatographed move down the column at speeds depending upon their partition coefficients between the mobile and stationary phases, as in countercurrent distribution. Column partition chromatography constitutes an essential part of the methods of Bauld (1956), of Givner et al. (1960b) and of Preedy and Aitken (1961b) for the determination of urinary oestrogens.

It has also been used by Engel et al. (1961), for the separation of urinary oestrogen metabolites following injection of radioactive precursors.

The further uses of column partition chromatography and countercurrent distribution will be discussed in the following section.

The different solvent systems which have been used for the separation of oestrogens in the methods discussed so far, together with the advantages and disadvantages of each method, have been presented by Preedy (1962).

The advent of thin-layer chromatography introduced a powerful and convenient tool for the separation of groups of closely related substances. Although there are limitations to its applicability, e.g. it is a small-scale process, it has already proved itself to be the most generally useful of the chromatographic techniques. The technique and its applications have been adequately reviewed by Truter (1963) and will, therefore, not be discussed further.

An extensive report on the use of thin-layer chromatography for the separation and characterisation of oestrogens has been presented by Lisboa and Diczfalusy (1962). Using six different solvent systems, they were able to separate by thin-layer chromatography on Silica Gel G a total of 24 steroid oestrogens. They also tabulated the R_f values for each of the oestrogens in the different solvent systems. The solvent systems studied

provided favourable resolutions of the various oestrogens, excellent reproducibility, and a high precision, as indicated by the fiducial limits of the various R_f values. Furthermore, the running time for free oestrogens was generally less than two hours. In a more recent publication, Lisboa and Diczfalusy (1963) have described a series of colour reactions for the *in situ* characterisation of steroid oestrogens on thin-layer chromatograms. In all, thirty-two steroid oestrogens were examined. The sensitivities of the different colour reactions ranged from 0.2-15.0 micrograms. These colour reactions have been developed specifically for the characterisation of oestrogens on thin-layer chromatograms.

Although it has been evident for some time that gas chromatography might be of great value in the separation and determination of steroid hormones, technical difficulties, particularly instability of steroids and certain steroid derivatives at high temperatures, have not been overcome until recently. The first practical demonstration of the separation of steroids by gas-liquid chromatography was described by VandenHeuvel *et al.* (1960a). This work was based upon the use of thin-film columns containing about 2% (w/w) of a highly thermostable nonselective phase (methyl silicone SE-30) coated on a diatomaceous earth support (80-100 mesh Chromosorb W). The operating temperature was about 220°. Retention times ranging from a few minutes to

an hour or more were observed for many steroids under these conditions. Since this report, the application of gas chromatography to the separation of urinary steroids has met with increasing success. Recently, the advances in gas chromatographic techniques to the study of human steroidal hormones have been reviewed by Horning et al. (1963).

The separation of the 'classical' oestrogens by gas chromatographic techniques was first described by VandenHeuvel et al. (1960b). Luukkainen et al. (1962) subsequently determined the relative retention times for a number of oestrogens with each of four types of liquid phases. This information may have applications in identification work. When quantitative work is attempted with free oestrogens, two effects are apparent. There is a partial loss of oestrogens, particularly oestriol, on the column and the retention time relationships are not entirely suitable for quantitative studies. It is not desirable to attempt to standardize conditions involving partial loss of a compound on a column; a better solution is to turn to derivatives. Furthermore, derivatives which are easily prepared are useful in identification work as well as for estimation procedures.

Acetates were proposed by Wotiz and Martin (1961, 1962), and by Fishman and Brown (1962) as suitable derivatives. The acetates are stable, easily prepared compounds. The

retention time relationships are such that either high temperatures or long retention times are involved, but with this limitation the method has given satisfactory results.

Luukkainen et al. (1962) have described the use of trimethylsilyl ether derivatives of oestrogens. This work was based upon the earlier observations of Luukkainen et al. (1961) on trimethylsilyl ethers. Oestrone, oestradiol-17β and oestriol trimethylsilyl ethers were separated with excellent resolution and with short retention times for all three derivatives.

Recently, Kroman and Bender (1963) have separated a mixture of oestrone, oestradiol-17β and oestriol at a column temperature of 243°. The retention time for oestriol was about eight minutes and there appeared to be no thermal instability as determined by examination of the chromatogram over an extended length of time, and by comparisons of the ultraviolet absorption spectra with reference spectra following the passage of the steroids through the column.

Wotiz (1963) has recently reported the separation and determination of 16-epioestriol (as the triacetate) in addition to the 'classical' oestrogens from urinary oestrogen extracts by gas chromatography.

4. IDENTIFICATION OF INDIVIDUAL OESTROGENS

Before the development of chromatographic techniques, the only means of identification was to show that the unknown

substance was identical with a known compound by the methods of classical organic chemistry. This was usually achieved by taking their melting points separately and mixed. The mixed melting point was an admirable check on identity, and a sharp melting point was a good criterion of purity: and this method has been justified largely in practice since Chevreul pioneered such use of melting points in 1823. Furthermore, this method can only be applied to substances which give definite melting points or else to those which are known to pass through different transition states or crystalline forms (e.g. oestrone, m.p. 254°, 256° and 259°C). In spite of the development of newer methods, crystallization is still the primary method for isolation. In some cases this may involve the processing of large amounts of material by chromatography before crystallization can be carried out (see Layne and Marrian, 1958b).

Chromatographic and isotopic tracer techniques made it possible to work with much smaller amounts of material. Although, under these conditions, absolute identification is difficult to achieve, it is possible to employ a series of additional identification procedures so that the authenticity of a particular compound becomes progressively more firmly established. In this respect, there are a number of identification procedures which can be used either separately or, preferably, together. The physico-chemical characteristics of the unknown substance

are compared with those of a known standard and if these are found to be the same by a number of different tests, the unknown and the standard may be considered identical. The procedures which have been used are: ultraviolet and infrared absorption spectroscopy; multiple chromatography of the free steroid and its derivatives; column partition chromatography and countercurrent distribution; and isotope dilution and reverse isotope dilution.

a. Ultraviolet and infrared absorption spectra.

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 number of steroids and steroid derivatives in various organic solvents. Zaffaroni (1950, 1953) 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 and Steyermark, 1955).

Infrared spectroscopy has become increasingly useful in providing information on the structure of steroid materials

(Jones and Herling, 1954). In the 'fingerprint' region, the absorption pattern tends to be characteristic of each individual steroid. Consequently, this region is particularly useful for identification purposes. Infrared spectroscopy has not been used extensively in the identification of oestrogens, mainly because of the very small amounts of oestrogens usually available from natural sources. However, modern instruments are available with attachments designed for very small samples, such as beam condensing units, and for KBr discs in which the unknown or standard can be incorporated. In this way, infrared spectra can be obtained on amounts of material of the order of 10 micrograms (Oertel et al. 1959).

b. Multiple chromatography.

Multiple chromatography can be used in many ways, with or without the addition of radioactive oestrogens. A single type of chromatography may be used, e.g. paper chromatography, with two or more different successive solvent systems. Comparison of the unknown and reference material is made each time. Alternatively, two or more types of chromatography may be used (Slaunwhite and Sandberg, 1956; Levitz et al. 1956; West et al. 1958a, b).

The derivatives of oestrogens most commonly used in identification of the parent steroid are monobenzoates, 3-methyl ethers and acetates. In practice, the untreated material is chromatographed successively in different solvent

systems and its chromatographic characteristics compared with that of the appropriate reference oestrogen. A derivative is formed and the procedure is repeated, comparison being made with the authentic oestrogen derivative.

Isotopes can be used in conjunction with the above procedure. The authentic radioactive material may be added to the inert material from an extract and the mixture then chromatographed. The chromatogram is then stained with a chemical reagent which has previously been shown to react in a similar fashion with the reference steroid. A radioautogram of the stained strip is then made. If the radioactive material, as located by radioautography and by the staining reaction superimpose, evidence of identity of the unknown with the radioactive reference has been obtained.

Successive chromatography of both the free compound and derivatives will provide further evidence of identity. The reverse of this technique, i.e. addition of authentic inert material to unknown radioactive material, can be used in the identification of metabolites following administration of a tracer dose of radioactive precursor (e.g. Ainsworth, Carter and Common, 1962).

A review of multiple paper chromatography as a means of identification of steroids together with a discussion on the limitations of this technique, has been presented by Bush (1961).

c. Countercurrent distribution and column partition chromatography.

In studies which involve the isolation of isotopically labelled substances by the addition and isolation of appropriately chosen unlabelled carrier compounds, or vice versa, the demonstration of the identity of the isotopic conversion product and the added carrier, or vice versa, is of primary importance. In this respect, countercurrent distribution and column partition chromatography are particularly valuable tools.

If an inert oestrogen and its radioactive counterpart are chromatographed together on a partition column, a typical Gaussian curve is obtained which can be outlined by measurement of radioactivity and by measurement of the inert oestrogen. After appropriate adjustments of scale, evidence of identity will have been obtained if the curves obtained by each method superimpose. The specific activity for each point is then determined and if this remains constant, within the limits of experimental error, one can assume that no separation of labelled from inert material has occurred. When countercurrent distribution is employed, it is advisable to use a large number of transfers (at least 72) for an efficient separation of the material under test from any interfering material. With either of these methods, the statistical probability of identity of the two compounds can be calculated from multiple analyses and

contains within it an evaluation of the effects of the experimental error in the determination of both parameters. It also gives a precise estimate of the specific activity, this quantity being indispensable for comparison with specific activities obtained after isotope dilution or reverse isotope dilution or other purification procedures. Furthermore, it is necessary to achieve a separation of the maxima of only one fraction to provide decisive and convincing evidence of radiochemical heterogeneity. A study of the use of countercurrent distribution as a means of determining radiochemical purity has been presented by Baggett and Engel (1957). The same arguments apply in the use of column partition chromatography.

d. Isotope dilution and reverse isotope dilution.

These techniques have provided valuable means of identification of materials from biological extracts and have been applied successfully in the identification of both inert and radioactive oestrogens.

The method of estimating or identifying an inert oestrogen by isotope dilution depends on the addition of a known number of counts of an authentic radioactive oestrogen to a sample containing the inert oestrogen; and secondly, on establishing a ratio in a sample aliquot between concentration of inert oestrogen on the one hand and counts on the other. From this, the amount of inert oestrogen

present in the original sample can be calculated by proportion. The oestrogen has to be separated efficiently from interfering material by chromatographic and extraction procedures as described above. The ratio between the inert and radioactive oestrogen is usually established either by countercurrent distribution or by column partition chromatography. Usually the average ratio in the peak tubes is taken, since it is usually assumed that radiochemical purity in the fractions at the top of the curve is adequate.

This method can be applied to the determination of oestrogens but it cannot be expected to provide any short cut to oestrogen estimations, since separation and extraction have to be carried out prior to isotope dilution. However, the value of this technique lies in the fact that, since it depends on the ratio of inert to radioactive oestrogen, extraction and separation losses do not affect the estimation.

Reverse isotope dilution is designed for the characterization of radioactive materials. Following extraction and separation of a radioactive oestrogen by the methods already described, a relatively large amount of an authentic inert oestrogen is added. The oestrogen fraction then consists of a small amount of radioactive oestrogen and a large amount of inert carrier. Radiochemical purity is then determined by repeated crystallization until the specific activity of the crystalline material and

the mother liquor remains constant. The amount of radioactive material present in the original sample can be determined by proportion, since the amount of inert oestrogen added is known.

The above techniques have been incorporated into elaborate procedures to establish clearly the identity of oestrogens in urine, usually in connection with metabolic experiments. These procedures have been of the utmost importance in providing definitive evidence of pathways of oestrogen metabolism, and have been employed widely in studies involving the administration of radioactive oestrogens to normal humans. Each study consists of a combination of the procedures previously described.

The design of sequential procedures to establish the identity of oestrogens beyond reasonable doubt depends on the particular end in view. The detailed procedures have been adequately covered in the texts cited in the preceding pages and are not here recapitulated.

CHAPTER II

MATERIALS AND METHODS

A. GENERAL MATERIALS AND APPARATUS

I. REFERENCE STANDARDS

Crystalline oestrone; obtained from Steroid Laboratories Ltd., Box 247, Montreal, Canada.

Crystalline 16-oxooestradiol-17β and crystalline 16-epioestriol; presented by Dr. D.S. Layne, Worcester Foundation for Experimental Biology, Shrewsbury, Mass., U.S.A.

Crystalline oestriol; presented by Dr. L. Blouin, Parke, Davis and Company, Detroit 32, Michigan, U.S.A.

Crystalline 16-epioestriol; presented by Dr. J. Fishman, Sloan-Kettering Institute, New York, N.Y., U.S.A.

Crystalline 16β-hydroxyoestrone diacetate; presented by Privat-Dozent Dr. H. Breuer, Chirugische Universitat-Klinik, Bonn, Germany.

Crystalline equol; presented by Dr. Wm. Klyne, Westfield College, London, N.W.3., England.

16-Oxooestradiol-17β-16-C^{14}; obtained from Dr. M. Levitz, Department of Obstetrics and Gynaecology, New York University, College of Medicine, New York, N.Y., U.S.A.

2. REAGENTS.

Absolute ethanol (Consolidated Alcohols Ltd., Toronto, Ontario) was purified by refluxing for twelve hours with 5% zinc dust and 5% sodium hydroxide pellets. The solution was then twice distilled.

Diethyl ether, U.S.P., was freed from peroxides according to the procedure of Bauld (1956) and distilled within twenty-four hours of use.

'Spectranalysed' grade methanol (Fisher Scientific Company, Montreal) was used for recrystallization. All other solvents were 'Analar' reagent grade (British Drug Houses Ltd., Toronto) and were distilled before use.

All reagents were analytical grade unless otherwise stated.

3. CHROMATOGRAPHIC MATERIALS

Hyflo-Supercel (Johns-Manville Company Ltd. (Canada), Montreal) was purified for chromatography as outlined by Engel et al. (1961).

Alumina, 'Woelm' neutral, activity grade 1, was obtained from Alupharm Chemicals, P.O. Box 755, New Orleans, La., U.S.A.

Whatman No. 1 and Whatman No. 42 filter paper strips were prepared for chromatography by continuous extraction in a Soxhlet apparatus with a mixture of methanol and benzene (1:1 v/v) and a mixture of methanol and chloroform

(1:1) respectively, for seventy-two hours. The papers were then allowed to air dry and stored in a dry place until required.

4. ENZYME PREPARATION.

The enzyme preparation used was a molluscan β-glucuronidase plus sulphatase 'Glusulase', prepared from the snail Helix pomatia and purchased from Endo Laboratories Inc., 84-40 10155 St., Richmond Hill, New York, N.Y., U.S.A. Each ml. of this preparation is standardized to contain 100,000 Fishman units of β-glucuronidase and 50,000 Fishman units of sulphatase.

5. COLOUR REACTIONS.

Detection of oestrogens on chromatograms was done by means of diazotised sulphanilic acid. The reagents were prepared and the colour developed as outlined by Axelrod and Pulliam (1960).

The Kober reaction was carried out according to Ittrich (1958). The procedure will be described later in Part 2 of this thesis.

6. APPARATUS.

Descending paper chromatograms were run in square glass tanks at room temperature or at 34°C, depending upon the system used.

Column partition chromatography was carried out by means of apparatus similar to that described by Engel et al. (1961). An automatic fraction collector, model 3V-4000, obtained from Buchler Instruments Inc., 514 West 147th Street, New York 31, N.Y., U.S.A., was used to collect 10 ml. fractions of eluent from the column.

Optical densities were measured with a Beckman Model DU spectrophotometer obtained from Beckman Instruments Inc., Fullerton, California, U.S.A.

Radioactivity was measured using a Packard Tri-Carb Liquid Scintillation Counter, Model 314EX, obtained from Packard Instrument Co. Inc., La Grange, Illinois, U.S.A.

Infrared spectrophotometry was carried out by Dr. R.N. Jones, Division of Pure Chemistry, National Research Council of Canada, Ottawa, using a Perkin-Elmer Model 21 Spectrophotometer fitted with a sodium chloride prism.

B. METHODS.

1. COLLECTION AND STORAGE OF URINE AND FAECAL SAMPLES.

Urine was collected from laying and non-laying hens which had been surgically modified by the technique of Dixon and Wilkinson (1957), so as to provide them with exteriorized ureters. This technique has been modified in certain details by the author in later work and will, therefore, be described in full in the Appendix. Alternatively, hens were surgically

modified by the technique of Richardson et al. (1960) so as to provide them with an artificial anus. The urine, collected twice daily, was either processed immediately a twenty-four hour sample had been collected, or kept at -20°C in polythene bottles in the deep freeze until required.

Twenty-four hour faecal samples were dispersed in 70% methanol and stored in the deep freeze until required.

2. HYDROLYSIS AND EXTRACTION OF OESTROGENS FROM URINE AND FAECES.

Acid and enzymic hydrolysis are the two main procedures used for liberating oestrogens from their conjugates. Acid hydrolysis of urine was carried out by diluting the urine sample, usually to 500 ml., followed by addition of 15 vol. per cent hydrochloric acid and subsequent hydrolysis by boiling under reflux for sixty minutes. The free oestrogens were then extracted and purified by the procedure of Bauld (1956) as outlined by Bauld and Greenway (1957), with the exception that column partition chromatography of the hydrophilic and lipophilic fractions was omitted. The purified fractions were each dissolved in a small amount of methanol.

Enzymic hydrolysis of urine was carried out using the procedure of Layne and Marrian (1958b). The urine sample, usually diluted to 500 ml. was adjusted to pH 4.8 by the addition of acetic acid followed by the addition of molar acetate buffer equal to one-fifth the volume of the urine. 'Glusulase' was added at the rate of 500 Fishman

units per ml. diluted urine, as recommended by Bugge et al. (1961) and the whole was then incubated at 37°C. for twenty-four hours. Before extraction with an equal volume of ether, Bradasol (a 5% solution of β-phenoxyethyldimethyldodecyl-ammonium bromide, manufactured by Ciba Ltd.) was added at the rate of 1 ml. per litre to facilitate the breaking of emulsions. A second extraction with the same volume of ether was made, this being added to the first extract. The ethereal extract of the enzymic digest was then 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.

Faecal samples were extracted with methanol prior to hydrolysis. The faeces when collected were dispersed in 70% methanol (300 ml.). This mixture was refluxed for twenty minutes and filtered with suction. The residue was re-extracted under reflux with a further 200 ml. of absolute methanol, filtered and the filtrate added to the first filtrate. The combined filtrates were evaporated in a rotary evaporator under reduced pressure until methanol had been removed. The aqueous solution was made up to 500 ml. with water and subjected to hydrolysis and extraction by the procedure of Bauld (1956) or that of Layne and Marrian (1958b) as outlined above. The purified phenolic fraction(s) was finally taken up in methanol.

3. MEASUREMENT OF RADIOACTIVITY.

a. Measurement of radioactivity in crude urine.

Daily urine samples were first filtered through a Büchner funnel with the aid of suction to separate the solid material. The latter was then ground up with 50 ml. distilled water and filtered. This filtrate was added to the first filtrate and the whole made up to 500 ml. with distilled water. The radioactivity present in each urine sample was then determined by the method of Flood et al. (1961). The diluted urine (0.6 ml.) was added to 6 ml. ethanol and 10 ml. of the usual scintillation fluid which contained 4 g. diphenyl oxazole (PPO) and 100 mg. 1,4-bis-2-(5-phenyl-oxazoyl) benzene (POPOP) per litre toluene. Duplicate samples were counted in a Packard Tri-Carb liquid scintillation counter at a thirty minute time setting. The contribution from C^{14} in the crude urine, corrected for quenching and efficiency, was then calculated as described by Okita et al. (1957). The quenching corrections were substantially the same for all the crude urine samples studied.

b. Measurement and distribution of radioactivity in urine.

The aqueous fractions that remained after enzymic hydrolysis and extraction of crude urine samples with ether, together with the acidic fractions (obtained by washing the ether fraction with 4 x 50 ml. 5% sodium carbonate) were made up to a known volume, usually 500 ml.,

and assayed for radioactivity as outlined for crude urine. The ether fractions, containing the phenolic material, were made up to 500 ml. with ether. Duplicate samples, each of 0.6 ml., were taken and assayed for radioactivity using a thirty minute time setting. The quenching correction was negligible in the case of the phenolic fractions.

c. Measurement and distribution of radioactivity in faeces.

Aqueous methanolic extracts of faeces were freed from methanol and were then assayed for radioactivity as outlined for crude urine. The aqueous, acidic and phenolic fractions were assayed in the same way as was the urine.

4. SEPARATION AND IDENTIFICATION OF OESTROGENS FOLLOWING EXTRACTION FROM URINE AND FAECES.

a. Chromatography.

The following paper chromatographic systems have been used in the present work to separate and purify oestrogen extracts:

(a) Benzene - 55% methanol

(b) Toluene - 70% methanol

(c) Absolute methanol - petroleum ether, b.p.80-100°C.

Systems (a) and (b) were used at room temperature, system (c) was used at 34°C, the paper being dried immediately before use as described by Layne et al. (1958). Equilibration and development of the chromatograms were carried out according to Bush (1952). The chloroform-formamide system as outlined by Layne and Marrian (1958b) was also used.

Inert oestrogens were located on chromatograms by staining with diazotised sulphanilic acid. Radioactive materials were located by exposure of chromatograms to X-ray film.

Column partition chromatography was carried out by gradient elution from a Celite column as outlined by Engel et al. (1961). The stationary phase was 70% methanol whilst the mobile phase was 2,2,4-trimethylpentane for the first 40 column volumes. This was then replaced by a gradient of 2,2,4-trimethylpentane and 1,2-dichlorethane for a further 80 column volumes.

Absorption chromatography on alumina was carried out according to Ittrich (1958). Alumina was deactivated by the addition of about 4 ml. H_2O to 100 g. alumina followed by shaking to get rid of lumps. Standardization of the alumina was carried out by preparing a 3 g. column containing 10 micrograms of authentic oestrone. The column was then eluted with 0.8% ethanol in benzene, the eluate being collected in 1 ml. fractions. Oestrone should begin to appear in the eluate after 12-14 ml. has passed through the column. Oestrone was detected by use of Ittrich's (1958) modification of the Kober reaction.

Extracts were applied to a 3 g. alumina column prepared in carbon tetrachloride with a mixture of 2 ml. benzene and 4 ml. carbon tetrachloride. The column was then washed with 5 ml. carbon tetrachloride followed by 5 ml. benzene. Following percolation of this, the column was

eluted with:-

(a) 9 ml. 0.8% ethanol in benzene. This fraction contains the quicker running steroids and part of the impurities.

(b) 10 ml. 0.8% ethanol in benzene. This fraction contains oestrone.

(c) 15 ml. 3% ethanol in benzene. This fraction contains oestradiol-17β.

(d) 15 ml. 20% ethanol in benzene. This fraction contains oestriol.

b. Identification of urinary oestrogens.

Following separation and purification of the urinary oestrogen fraction by the above chromatographic procedures, either alone or in sequence, the identity of the particular oestrogens had to be established beyond reasonable doubt. Inert materials were further purified by repeated crystallization from one or more solvents. The recrystallized material was then subjected to infrared analysis and its infrared spectrum was compared with that of an authentic reference standard.

Radioactive oestrogens were identified by a sequence of steps involving paper chromatography in sequence after addition of authentic reference material, reverse isotope dilution and repeated crystallization to constant specific activity from aqueous methanol of both the free oestrogen and a derivative.

5. DERIVATIVE FORMATION.

a. Acetylation.

The material to be acetylated was dissolved in 0.4 ml. pyridine, 0.2 ml. acetic anhydride was added and the mixture was left overnight at room temperature. The mixture was then poured into 100 ml. iced water containing 5 ml. 6N sulphuric acid. The aqueous mixture was extracted with ethyl acetate (1 x 100 ml; 2 x 50 ml.). Pyridine was removed by washing the ethyl acetate extract five times with 5 ml. 6N sulphuric acid. The ethyl acetate was then washed three times with 10 ml. N NaOH and finally three times with 5 ml. distilled water. The acetate derivative was recovered by evaporation of the ethyl acetate. The melting point of the acetate derivative was determined with a Fisher-Johns Melting Point Apparatus (Fisher Scientific Co., Montreal, Canada).

b. Sodium borohydride reduction.

The material to be reduced was dissolved in ethanol. Water was added to give an ethanol concentration of about 50% (v/v). After addition of sodium borohydride (10 mg. per ml.), the mixture was shaken intermittently by hand and then allowed to stand in the dark overnight. Excess borohydride was destroyed by the careful, dropwise addition of 10% acetic acid. The reduced compounds were then extracted with 8.5% sodium bicarbonate. The ether was finally evaporated and the residue dissolved in methanol.

CHAPTER III

ISOLATION OF OESTRONE AND OF EQUOL FROM THE URINE OF THE DOMESTIC FOWL

A. INTRODUCTION.

Although oestriol, oestradiol and oestrone have been detected in mixed avian excreta (Hurst et al. (1957) and in extracts of ovaries of laying hens (Layne et al. 1958) by chromatographic methods, it would appear that only oestradiol-17β has been isolated hitherto from avian excreta in crystalline form and identified by infrared spectroscopy (MacRae et al. 1959).

The experiment described below was designed to isolate the naturally occurring oestrogens from urine in amounts such that they could be identified by physico-chemical methods.

B. METHOD.

A laying hen was surgically modified so as to provide it with exteriorised ureters. Urine was collected for a total of 128 days, during which time approximately forty litres of diluted urine was obtained. This urine was subjected to acid hydrolysis in one-litre batches and the oestrogen extracts thereof were prepared and purified by the method of Bauld (1956) as outlined by Bauld and Greenway (1957). The hydrophilic and lipophilic fractions from each

batch were then chromatographed in the chloroform-formamide system. After development of the chromatograms, strips 1 cm. in width were cut from each side of the paper and the positions of the oestrogens on these strips were located by diazotised sulphanilic acid reagent. The appropriate areas were then cut out from the main part of each paper and extracted with a mixture of methanol-dichloromethane (1:1 v/v). After evaporation of the extracts, formamide was removed from the eluted fractions by distribution between ether and water; and the ether extracts were dried over sodium sulphate and evaporated to dryness.

C. RESULTS.

Chromatography of the lipophilic extracts resulted in fractionation into three distinct zones having mobilities corresponding to equol, oestradiol-17β and oestrone. The presumptive oestrone and oestradiol-17β fractions obtained by eluting the appropriate areas of the papers were each purified further by chromatography on alumina as described by Ittrich (1958). After removal of the solvent, each fraction was recrystallized twice from aqueous ethanol. At this stage the final yield of material from the oestrone fraction was approximately 0.25 mg. and that from the oestradiol-17β fraction was approximately 0.2 mg.

Each fraction was dissolved in acetone and the solutions evaporated on powdered potassium bromide, which

was then ground by hand and pressed into discs. Samples of authentic reference oestrone and of oestradiol-17β were similarly prepared. The infrared absorption spectrum of each of the samples was then determined.

The infrared absorption spectra of the presumptive oestrone from hen's urine and of the reference oestrone are shown in Figure 2. There were differences in the region of the -OH stretching bands, but these were probably a consequence of differences in crystallinity or water absorptivity as between the samples. There was excellent agreement between the two spectra for all points below 2000 cm^{-1}. Accordingly, it may be concluded that the crystalline material isolated from hen's urine was identical with reference oestrone.

In the case of the spectra of the presumptive oestradiol-17β from hen's urine and of the reference oestradiol-17β, these were not identical and the lack of correspondence was specially noticeable in the 'fingerprint' region. On this evidence, it may be concluded that the crystalline material isolated from avian urine did not represent pure oestradiol-17β.

Staining of the chromatograms from the hydrophilic fractions revealed a very polar material having a mobility corresponding to that of oestriol and a very diffuse less polar zone corresponding in mobility to equol and 16-epioestriol. Accordingly, the presumptive oestriol zones

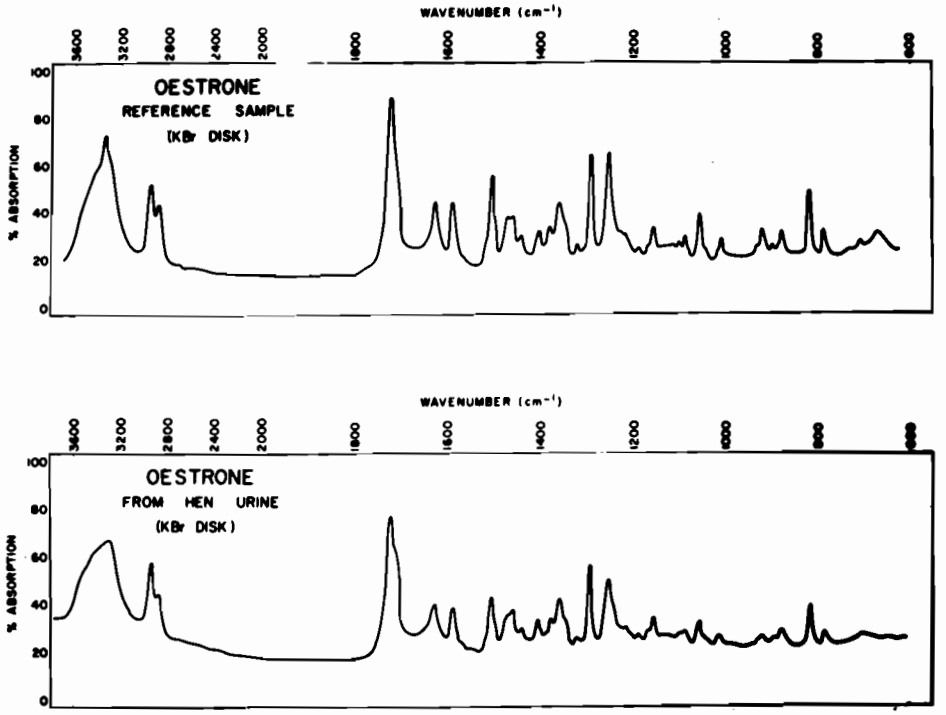


FIGURE 2. COMPARISON OF INFRARED ABSORPTION SPECTRA OF REFERENCE CRYSTALLINE OESTRONE (UPPER FIGURE) AND OF CRYSTALLINE OESTRONE (LOWER FIGURE) ISOLATED FROM THE URINE OF A LAYING HEN.

were cut out and eluted and the residue applied to an alumina column. The oestriol fraction was evaporated, but attempts to obtain crystalline material from the yellow oil which resulted were unsuccessful. The less polar zones on the chromatograms were not examined further.

At this point attention was directed to the more polar material present on the chromatograms of the lipophilic extracts. This material had a mobility corresponding to that of equol. The presumptive equol zones were cut out and eluted. The solvent was evaporated and the residue at this stage proved to be semicrystalline. Ths residue was then re-crystallised twice from benzene and three times from aqueous methanol. The final yield of crystals was approximately 0.43 mg.

The entire sample was dissolved in acetone and the solution was evaporated on powdered KBr, which was then ground and pressed into a disc for infrared spectrophotometry. A sample of reference equol was similarly prepared. The infrared absorption spectra (Figure 3) of the avian material and of the reference equol showed substantial agreement for all points below 2000 cm^{-1}. There were differences in the region of the -OH stretching bands at 2500-3300 cm^{-1}, which again could have been a consequence of differences in crystallinity or water absorptivity as between the two samples. However, the agreement of the spectra of the two samples in the 'fingerprint' region

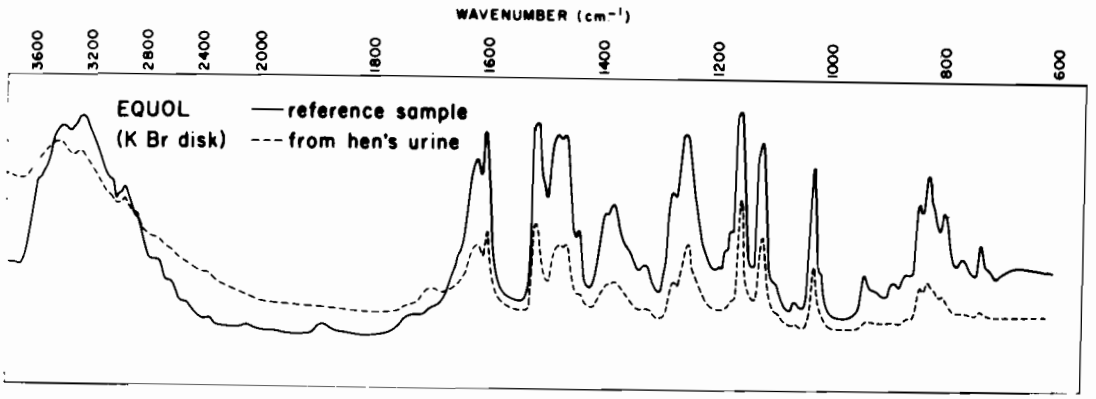


FIGURE 3. COMPARISON OF INFRA RED ABSORPTION SPECTRA OF CRYSTALLINE REFERENCE EQUOL AND OF CRYSTALLINE EQUOL ISOLATED FROM THE URINE OF A LAYING HEN.

(1300-700 cm^{-1}) provided strong evidence that the crystalline material isolated from avian urine was identical with equol.

D. DISCUSSION.

The results of this investigation permit the conclusion that the oestrogenic compound isolated in crystalline form from lipophilic fraction of the phenolic fraction of urine from laying hens was identical with oestrone.

The isolation of oestrone, and the failure to isolate oestradiol-17β and oestriol, would seem to suggest that oestrone may be the main oestrogenic hormone excreted in this species. The presence of minute quantities of oestradiol-17β and oestriol, however, could not be excluded on the basis of the present study. No attempt was made to isolate material from the 16-epioestriol zone of the hydrophilic fraction due to its being contaminated with equol.

The isolation of equol from the urinary lipophilic phenolic fraction is not surprising in view of the fact that the hen's diet was a commercial layers' ration containing dehydrated alfalfa meal. It is known that many fodder plants contain oestrogenic substances. Some of these are isoflavones, e.g. genistein, biochanin A and diadzein, and at least one is a fairly closely related substance, coumestrol (Kohler and Bickoff, 1961; Bickoff, 1961). These compounds are believed to undergo metabolism in the body giving rise to equol. It has recently been shown that at least one of these compounds, genistein, does, in fact, give rise to equol in the urine

of the domestic fowl (Cayen and Common, 1963). The isolation of equol from the urine of the goat (Klyne and Wright, 1957) and of the cow (Klyne and Wright, 1959) has also been reported.

CHAPTER IV

STUDIES ON THE INTERCONVERSIONS OF RING D DISUBSTITUTED OESTROGENS BY THE DOMESTIC FOWL.

I. THE IDENTIFICATION OF URINARY AND FAECAL CONVERSION PRODUCTS FOLLOWING INJECTION OF 16-OXOOESTRADIOL-17β-16-C^{14}.

A. INTRODUCTION.

Certain in vivo experiments on the interconversions of oestrogens in the fowl have been reported from this laboratory. In earlier experiments, MacRae and Common (1959, 1960) obtained chromatographic evidence for the presence of radioactive oestriol, 16-epioestriol, oestradiol-17β and oestrone in the excreta of hens that had received an intravenous injection of oestradiol-17β-16-C^{14}. Ainsworth, Carter and Common (1962) obtained similar evidence for the presence of the following radioactive conversion products in the urine of hens that had received an intravenous dose of oestrone-16-C^{14}:- oestriol, 16-epioestriol, 17-epioestriol, 16-oxooestradiol-17β, oestradiol-17β and 16-oxooestrone. It was further demonstrated that the laying hen was also capable of converting oestriol into 16-oxooestradiol-17β and 16-epioestriol, as evidenced by the chromatographic identification of the latter radioactive conversion products in the urine of hens which had received an intravenous dose

of oestriol-16-C^{14} (MacRae et al. 1960). Levitz et al. (1958) had previously shown that this conversion could take place in vivo in the human species. Levitz et al. (1960) later demonstrated that 16-oxooestradiol-17β could be reduced in vivo in the human subject to form oestriol and 16-epioestriol.

In view of the foregoing results, it became desirable to study the interconversions of 16-oxooestradiol-17β and of 16-epioestriol in the fowl, particularly in view of the fact that 16-epioestriol had appeared to be a more prominent urinary excretory product than oestriol in the experiments so far performed (MacRae and Common, 1960; Ainsworth et al. 1962).

B. METHOD.

16-Oxooestradiol-17β-16-C^{14} was purified by filter paper chromatography in the benzene-55% methanol system. Following exposure of the chromatographs to X-ray film, the radioactive zones corresponding to 16-oxooestradiol-17β were eluted and used in the studies. The purity of the preparation was checked by paper chromatography in the chloroform-formamide system. The specific activity was approximately 22 microcuries per mg.

The 16-oxooestradiol-17β-16-C^{14} in 2 ml. propylene glycol was injected intravenously into non-laying White Leghorn hens which had been surgically modified so as to provide them with artificial ani. Urine and faeces voided subsequently to the injection were collected separately for

several days as described below and stored as described previously.

C. EXPERIMENTAL AND RESULTS.

1. EXPERIMENTS 1 AND 2.

16-Oxooestradiol-17β-16-C^{14} was administered to two White Leghorn non-laying hens in the manner described above. The urines for the succeeding twenty-four hour periods were collected and subjected to enzymic hydrolysis. The ether extracts which contained the phenolic fractions were assayed for radioactivity with the results shown in Table II. The greater part of the radioactivity recovered in the phenolic fraction of the urine was recovered within the first twenty-four hours following injection. Excretion of radioactivity on subsequent days was relatively small.

Following purification of the phenolic fractions, the oestrogen extracts for the first twenty-four hour periods were subjected to preliminary examination by chromatography in the chloroform-formamide system. Radioautography revealed the presence of two radioactive zones. These zones corresponded, on the basis of mobilities, to 16-epioestriol and 16-oxooestradiol-17β, the more polar being the presumptive 16-epioestriol. No radioactive zone corresponding to oestriol was evident.

These two radioactive zones (Bird No. 1 only) were then eluted separately from the chromatogram with methanol.

TABLE II

Excretion of radioactivity in the phenolic fraction from the urine of non-laying hens following intravenous injection of 16-oxooestradiol-17β-16-C^{14}

Bird No.	Amount injected c.p.m.	Daily excretion*		
		1st day	2nd day	3rd day
1	5.44×10^5	17.1	5.0	Not measured
2	2.72×10^5	12.0	3.2	2.8

*Expressed as % injected radioactivity.

After evaporation of the methanol, the two fractions were rechromatographed in the absolute methanol-petroleum ether system. The presumptive 16-oxooestradiol-17β was accidentally lost during chromatography. The presumptive 16-epioestriol zone was eluted from the chromatogram, divided into two parts and tested as to identity by chromatography in admixture with cm. 25 micrograms of authentic reference 16-epioestriol in the systems chloroform-formamide and toluene-70% methanol. The radioautogram and the stained chromatograms of the 16-epioestriol zones were identical as to shape and position in each case (Figure 4), thereby suggesting that the radioactive material on the chromatograms was identical with the reference 16-epioestriol added.

2. EXPERIMENT 3.

In view of the foregoing preliminary results, a third experiment was performed in which the isolated radioactive conversion products were examined for radiochemical purity. The quantitative and qualitative aspects of the conversion were also evaluated.

16-Oxooestradiol-17β-16-C^{14} (0.26 mg.) was administered to a White Leghorn non-laying hen as described above. Urine and faeces were collected separately for the succeeding seven days following injection. The crude urines for each twenty-four hour period were assayed separately for total radioactivity, and subjected to hydrolysis and ether extraction. Each extract was then subjected to preliminary

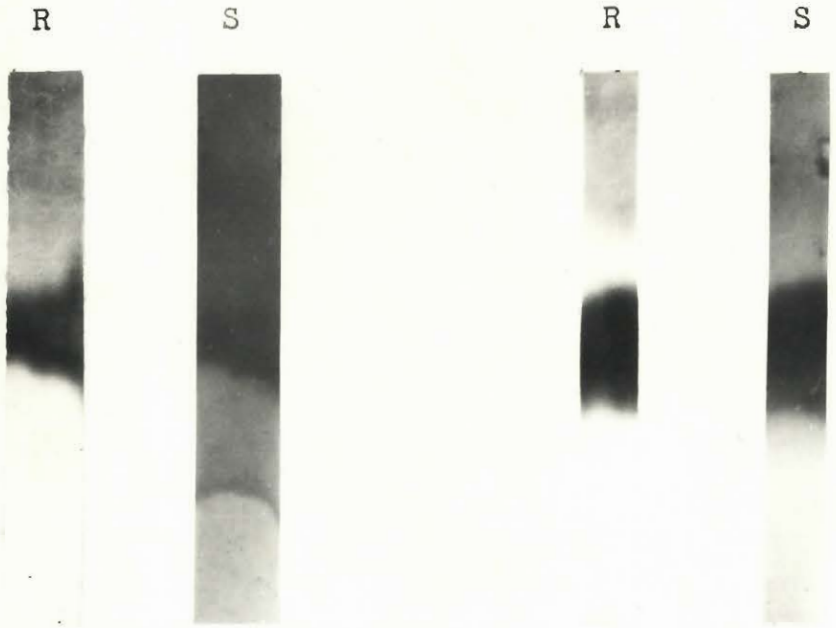


FIGURE 4. CHROMATOGRAPHIC IDENTIFICATION OF 16-EPIOESTRIOL FROM THE URINARY EXTRACT OF A NON-LAYING HEN AFTER INJECTION OF 16-OXOOESTRADIOL-17β-16-C^{14}.

1.R. Radioautogram of chromatogram of presumptive 16-epioestriol zone after admixture with 25 micrograms of reference 16-epioestriol and chromatography in the system chloroform-formamide.

1.S. Chromatogram following staining with diazotised sulphanilic acid.

2.R. Radioautogram of a similar mixed chromatogram after chromatography in the system benzene-55% methanol.

2.S. Chromatogram following staining with diazotised sulphanilic acid.

purification. The faeces for the entire period were bulked, and the aqueous extract thereof assayed for radioactivity. The aqueous extract was then subjected to hydrolysis, extraction and preliminary purification. The recoveries and distributions of radioactivity in the phenolic, acidic and aqueous fractions from urine and faeces are presented in Table III. The radioactivity in the urine declined after the first day as a logarithmic function of time (Figure 5).

a. Initial purification and separation of urinary and faecal phenolic fractions.

The urinary phenolic fractions from days 1, 2 and 3 were subjected separately to initial purification and separation by chromatography in the benzene-55% methanol system. The urinary phenolic fractions from days 4, 5 and 6 were bulked and similarly chromatographed. After chromatography and exposure to X-ray film the different zones of radioactivity were cut out and eluted with methanol. Radioautograms of the chromatograms of the urinary phenolic fractions are shown in Figure 6. The most polar zone (R_f = 0.32) appeared only on the chromatogram from the first day. The radioactive zone corresponding to 16-oxooestradiol-17β appeared only on the chromatograms for the first and second days. Thereafter, most of the radioactivity was present in a single zone having a mobility corresponding to that of 16-epioestriol. The faecal phenolic fraction was similarly chromatographed.

TABLE III

Recoveries and distributions of radioactivity in the urine and faeces of a non-laying hen following intravenous injection of 16-oxooestradiol-17β-16-C^{14} (7.6×10^6 c.p.m.).

Collection Period (days)	Radioactivity in crude urine and/or faeces (c.p.m.)	Distribution of radioactivity in the various fractions		
		Aqueous (c.p.m.)	Acidic (c.p.m.)	Phenolic (c.p.m.)
		(a) Urine		
1	1,920,000 (25.3)*	317,000 (16.5)+	125,000 (6.6)+	1,200,000 (62.6)+
2	372,000 (4.9)*	37,400 (10.1)+	6,700 (1.8)+	282,000 (75.9)+
3	217,000 (2.8)*	15,400 (7.1)+	4,470 (2.1)+	159,000 (73.4)+
4	172,000 (2.3)*	16,700 (9.7)+	2,230 (1.3)+	127,000 (73.5)+
5	99,100 (1.3)*	7,550 (7.6)+	negligible	62,500 (63.1)+
6	48,800 (0.64)*	2,360 (4.8)+	negligible	20,800 (42.7)+
7	34,500 (0.45)*	not counted	not counted	not counted
Total	2,863,400 (37.7)*	396,410 (14.1)+	138,400 (4.9)+	1,851,300 (65.6)+
		(b) Faeces		
1 - 7	158,000 (2.1)*	23,600 (14.9)**	6,550 (4.1)**	122,000 (77.1)**

* Recovery of radioactivity expressed as % radioactivity injected.

\+ Recovery of radioactivity expressed as % radioactivity in crude urine.

** Recovery of radioactivity expressed as % radioactivity in faeces.

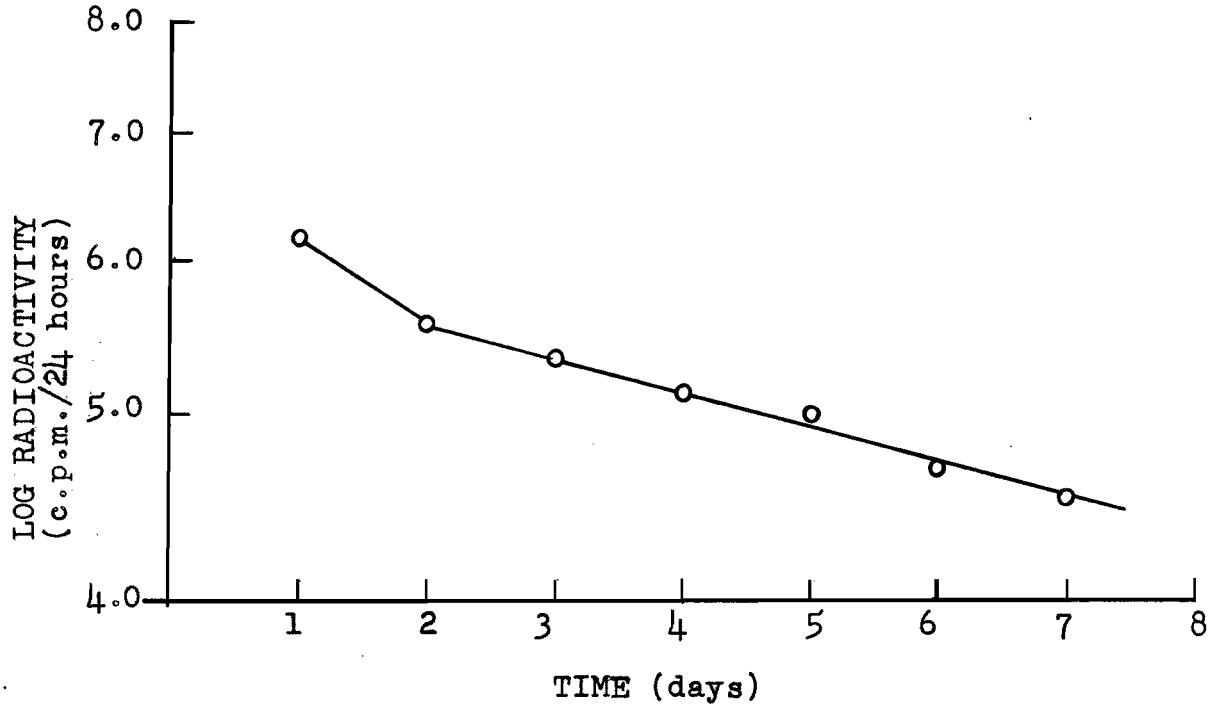


FIGURE 5. Plot of logarithm of radioactivity against time following injection of 16-oxooestradiol-17β-16-C^{14} into a non-laying hen.

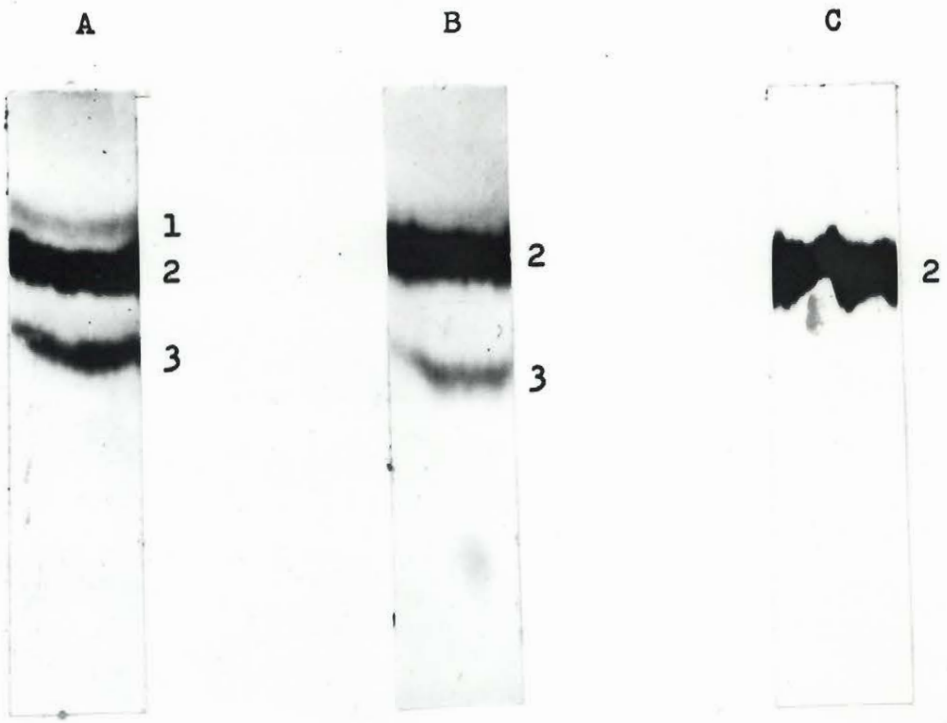


FIGURE 6. RADIOAUTOGRAMS OF CHROMATOGRAMS* OF THE PHENOLIC EXTRACTS FROM 24-HOUR URINE SAMPLES FOLLOWING INJECTION OF 16-OXOOESTRADIOL-17β-16-C^{14}.

A. Radioautogram of the first 24-hour urine extract.

B. Radioautogram of the second 24-hour urine extract.

C. Radioautogram of pooled extracts from the third, fourth and fifth 24-hour urine samples.

NOTE: Zone 1 was not identified; zone 2 was subsequently identified as 16-epioestriol; zone 3 was subsequently identified as 16-oxooestradiol-17β.

*System benzene-55% methanol.

Radioautography revealed the presence of only one zone, which had a mobility corresponding to that of 16-epioestriol. This zone was cut out and eluted with methanol. The eluates from corresponding zones from the urinary chromatograms were bulked, made up to a definite volume, and assayed for radioactivity. The eluate from the faecal chromatogram was similarly assayed. The recoveries and distributions of radioactivity between the chromatographic zones are shown in Table IV.

Following initial chromatographic purification of the urinary fraction, the 16-oxooestradiol-17β fractions (188,300 c.p.m.), an aliquot from the 16-epioestriol fraction (399,400 c.p.m.) and the unknown fraction (45,900 c.p.m.) were bulked together, the methanol evaporated, and the residue applied to a Celite gradient elution column with ca. 100 micrograms each of oestriol, 16-epioestriol and 16-oxo-oestradiol-17β carrier. Alternate tubes from the column were assayed for radioactivity, and in areas of concentration of radioactivity each tube was assayed. The distribution and recovery of radioactivity from the column are illustrated in Figure 7 and Table V. There is an apparent discrepancy in Table V between the proportions of radioactivity in the fraction as applied to the column and as recovered after elution, although the total recovery from the column was practically quantitative. This discrepancy was almost certainly due to the fact that the fractions as

TABLE IV

Preliminary paper chromatography of the 'phenolic' radioactivity extracted from urine and faeces of a non-laying hen following intravenous injection of 16-oxooestradiol-17β-16-C^{14}

	R_f*	Distribution of radioactivity**		Chromatographic identification
		c.p.m.	per cent	
a) Urine	0.32	45,900	3	?
	0.37	1,190,700	84	16-epioestriol
	0.48	188,300	13	16-oxooestradiol-17β
Total		1,424,900	100	
b) Faeces	0.38	102,100	100	16-epioestriol

* Benzene - 55% methanol.

**Distribution is approximate because of some overlap of zones.

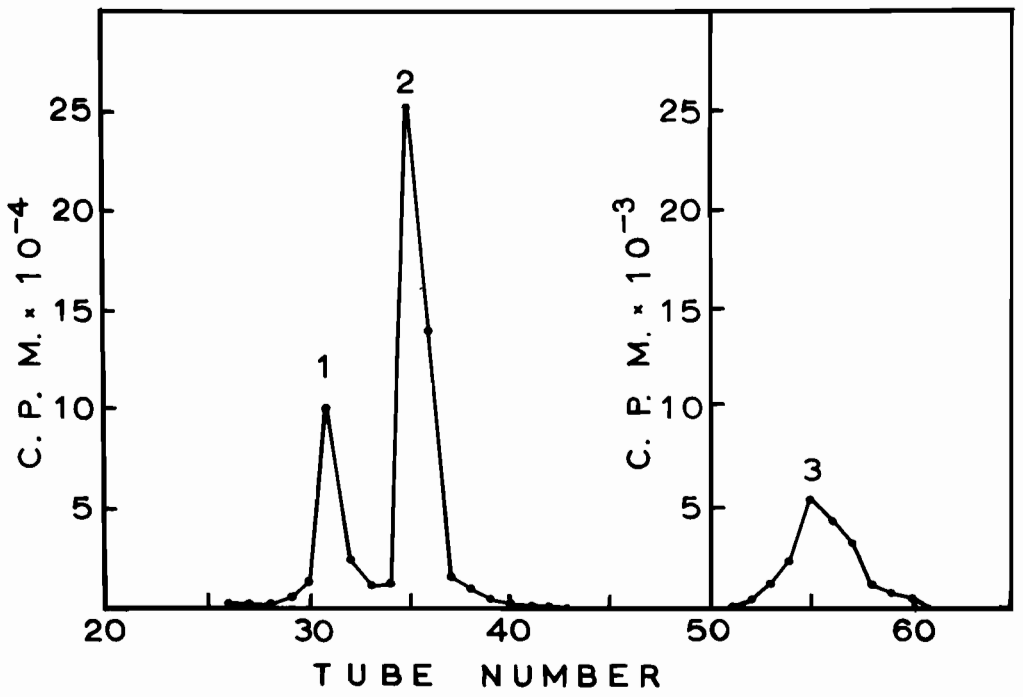


FIGURE 7. Distribution of radioactivity from a Celite column. Stationary phase was 90% methanol; mobile phase was 2,2,4-trimethylpentane for the first forty column volumes and was then replaced by a gradient of 2,2,4-trimethylpentane and 1,2-dichloroethane (1:1). All radioactivity was eluted into tubes numbered from the application of the gradient as shown. Each tube represents an elution volume of 10 ml. (column volume 9.0 ml.).

TABLE V

Recovery of radioactivity from a Celite partition column

(Details as for Fig. 7)

Tube No.	Radioactivity (c.p.m.)	Identification
26-33. (31)*	157,950	16-oxooestradiol-17β
34-41. (35)	446,850	16-epioestriol
53-60. (55)	18,900	not identified
Total	623,700	

*Figure in brackets indicates the tube containing the peak radioactivity.

eluted from the preliminary paper chromatographic zones were not in fact chromatographically pure because of overlap.

The distribution of carrier oestrogens in areas of concentration of radioactivity was assayed by the Ittrich (1958) modification of the Kober reaction. The peaks of radioactivity corresponded closely to the elution of 16-oxooestradiol-17β (peak 1), 16-epioestriol (peak 2) and oestriol (peak 3) from such columns. This was verified by the fact that the distribution of radioactivity and carrier oestrogen coincided in the case of peaks 1 and 2. No carrier oestriol was detected in the peak tubes of peak 3.

b. Purification and identification of radioactive 16-oxooestradiol-17β from urine.

The 16-oxooestradiol-17β peak tubes (Nos. 30-32) from the column were pooled and run successively in two chromatographic systems. In each instance the bulk of the radioactivity ran with the carrier 16-oxooestradiol-17β but the specific activity dropped slightly. The pool recovered from the chromatograms was then crystallized to constant specific activity following addition of 47.2 mg. reference crystalline 16-oxooestradiol-17β. Both crystals and mother liquors were analysed. After the third crystallization, the crystals and mother liquor from this third crystallization, together with a further 5.0 mg. reference 16-oxooestradiol-17β, were acetylated and again recrystallized

to constant specific activity. A summary of the recoveries and specific activities for the above purification steps is presented in Table VI.

c. Purification and identification of radioactive 16-epioestriol from urine.

The radioactive 16-epioestriol fraction was purified in a manner similar to that described for the radioactive 16-oxooestradiol-17β. The recoveries and specific activities for the purification steps are summarised in Table VII.

d. Purification and identification of radioactive 16-epioestriol from faeces.

After preliminary chromatography, ca. 100 micrograms of reference 16-epioestriol was added to the radioactive material and the mixture was then chromatographed successively in two paper chromatographic systems. In each case the bulk of the radioactivity ran with the 16-epioestriol carrier. Reference crystalline 16-epioestriol (6.6 mg.) was added to the 16-epioestriol pool from the second chromatography. Crystallization to constant specific activity was then carried out, both crystals and mother liquors being analysed. Both the crystals and the mother liquor from the first crystallization had essentially the same specific activity and there was no subsequent change on further recrystallization (see Table VIII). After the second crystallization, the mother liquors and crystals from the two crystallizations were bulked, the solvent evaporated and the whole acetylated.

TABLE VI

A. Purification of radioactive 16-oxooestradiol-17β from the urine of a non-laying hen.

Purification Step	c.p.m.	µg.	Specific Activity (c.p.m./µg.)
Pool off Celite Column (tubes 30-32)	126,500	64.4*	1964
Paper chromatography			
Benzene - 55% methanol	94,200	45.4*	2075
Toluene - 70% methanol	71,200	39.1*	1821

B. Crystallization of 16-oxooestradiol-17β to constant specific activity.

Purification Step	c.p.m.	mg.	Specific Activity (c.p.m./mg.)
Original pool	59,232	47.2	1255
1st crystallization	39,600	33.5	1182
Mother liquor	15,150	13.0	1165
2nd crystallization	23,000	19.15	1201
Mother liquor	12,900	10.46	1233
3rd crystallization	14,200	11.61	1220
Mother liquor	7,350	5.97	1231

C. Crystallization of 16-oxooestradiol-17β diacetate** to constant specific activity

Purification Step	c.p.m.	mg.	Specific Activity (c.p.m./mg.)
Original pool	19,250	27.89	690
1st crystallization	11,400	16.54	689
Mother liquor	6,800	9.88	688
2nd crystallization	3,400	5.01	680
Mother liquor	6,100	9.08	672

*Determined by Ittrich's (1958) modification of the Kober reaction.

**M.P. 132.5 - 133.5°C (uncorrected).

TABLE VII

A. Purification of radioactive 16-epioestriol from the urine of a non-laying hen.

Purification Step	c.p.m.	µg.	Specific Activity (c.p.m./µg.)
Pool off Celite column (tubes 35-37)	222,150	38.2*	5827
Paper chromatography			
Benzene - 55% methanol	178,450	25.4*	7028
Toluene - 70% methanol	141,400	23.8*	5940

B. Crystallization of 16-epioestriol to constant specific activity.

Purification Step	c.p.m.	mg.	Specific Activity (c.p.m./mg.)
Original pool	23,338	44.1	529
1st crystallization	10,750	20.4	527
Mother liquor	11,950	22.6	529
2nd crystallization	5,200	10.17	511
Mother liquor	4,550	8.98	506

C. Crystallization of radioactive 16-epioestriol triacetate to constant specific activity.

Purification Step	c.p.m.	mg.	Specific Activity (c.p.m./mg.)
Original pool**	9,050	26.97	336
1st crystallization	5,750	16.73	344
Mother liquor	2,650	8.37	317
2nd crystallization	3,600	10.64	338
Mother liquor	900	2.81	320

*Determined by Ittrich's (1958) modification of the Kober reaction.

**Crystals and mother liquor from the 2nd crystallization subjected to the acetylation procedure. M.P. 149.5 - 150.5°C (uncorrected).

TABLE VIII

A. Purification of radioactive 16-epioestriol from the faeces of a non-laying hen.

Purification Step	c.p.m.	µg.	Specific Activity (c.p.m./µg.)
Paper chromatography			
Benzene - 55% methanol	84,850	71.9*	1180
Toluene - 70% methanol	68,000	59.4*	1145

B. Crystallization of radioactive 16-epioestriol to constant specific activity.

Purification Step	c.p.m.	mg.	Specific Activity (c.p.m./mg.)
Original pool	5,616	6.6	851
1st crystallization	3,400	3.95	861
Mother liquor	2,300	2.73	842
2nd crystallization	1,800	2.15	836
Mother liquor	1,150	1.38	834

C. Crystallization of radioactive 16-epioestriol triacetate to constant specific activity.

Purification Step	c.p.m.	mg.	Specific Activity (c.p.m./mg.)
Original pool**	4,550	9.22	482
1st crystallization	2,800	5.92	473
Mother liquor	1,600	3.50	457
2nd crystallization	1,450	3.09	469

*Determined by Ittrich's (1958) modification of the Kober reaction.

** Crystals and mother liquors from both crystallizations subjected to the acetylation procedure. M.P. 149 - 150°C (uncorrected).

The 16-epioestriol triacetate that resulted from the acetylation was then recrystallized to constant specific activity. A summary of the recoveries and specific activities for the above purification steps is presented in Table VIII.

e. Attempt to identify the third fraction from the partition chromatogram.

The contents of tubes 52-60 were pooled, the solvent was evaporated and the residue was chromatographed in the toluene - 70% methanol system. The radioactivity was concentrated in a single zone on the chromatogram. This zone was cut out and eluted and crystalline oestriol (50.1 mg.) was added to the eluate. The total radioactivity present at this stage was 19,298 c.p.m. (sp. activity 385 c.p.m./mg.). After the first crystallization from aqueous methanol, the specific activity of the crystals dropped to 147 c.p.m./mg. After the second crystallization, the specific activity of the crystals dropped to 73 c.p.m./mg. and after a third crystallization it dropped to 28 c.p.m./mg. Clearly the radioactivity was mainly, if not entirely, due to material other than oestriol.

D. DISCUSSION.

The two preliminary experiments suggested that 16-oxooestradiol-17β injected into the fowl gave rise to urinary 16-epioestriol and no other urinary conversion product. The results of the third, and main experiment, provided substantial

confirmation of this finding, the confirmation being based on purification of urinary radioactive 16-oxooestradiol-17β and radioactive 16-epioestriol to radiochemical purity. In this experiment a third minor radioactive urinary constituent was observed but not identified; appropriate purification experiments showed that little, if any, of it was identical with oestriol.

The results of the preliminary paper chromatography (Table IV) showed that the amount of radioactive 16-oxo-oestradiol-17β in the urine was approximately one-sixth as much as the amount of radioactive 16-epioestriol. After the first forty-eight hours following injection, radioactive 16-oxooestradiol-17β could not be detected on the urinary chromatograms. These facts suggest a high efficiency of conversion of 16-oxooestradiol-17β to 16-epioestriol in the fowl.

Levitz, Rosen and Twombly (1960) have demonstrated that when 16-oxooestradiol-17β is converted in the human to oestriol and 16-epioestriol, then oestriol is the predominant reduction product. In vitro experiments by Breuer, Nocke and Knuppen (1958) have shown that human liver slices reduce 16-oxooestradiol-17β to oestriol and 16-epioestriol, the ratio oestriol : 16-epioestriol being approximately 2 : 1.

The present results suggest that the conversion of 16-oxooestradiol-17β in the fowl differs from that in the human in so far as the conversion product in the fowl is

almost exclusively 16-epioestriol. However, this point will be discussed further in connection with the study of the conversion products of 16-epioestriol reported below.

The only radioactive conversion product recovered from the faeces was 16-epioestriol.

II. THE IDENTIFICATION OF URINARY CONVERSION PRODUCTS FOLLOWING INJECTION OF 16-EPIOESTRIOL-16-C^{14}.

A. PREPARATION OF 16-EPIOESTRIOL-16-C^{14}.

16-Oxooestradiol-17β was purified as described above. The purified material was then dissolved in ethanol and reduced with sodium borohydride. Reduction of 16-oxooestradiol-17β in this way gives rise to 16-epioestriol in almost quantitative yield (Huffman and Lott, 1955).

The reduced material was purified by chromatography in the system benzene-55% methanol. The 16-epioestriol on the chromatograms was located by exposure of the chromatograms to X-ray film. The 16-epioestriol was then eluted from the chromatograms with methanol.

The purity of the product was checked by running aliquots with 20 micrograms of reference 16-epioestriol in the system chloroform-formamide. Radioautography revealed a single radioactive spot which ran with the added carrier 16-epioestriol.

The 16-epioestriol thus prepared had a specific activity of approximately 20 microcuries per mg.

B. METHOD.

16-Epioestriol-16-C^{14} in 2 ml. propylene glycol was injected intravenously into two White Leghorn non-laying hens which had been surgically modified so as to provide them with exteriorised ureters. The first bird received 0.23 mg. 16-epioestriol (6.8×10^6 c.p.m.) and the second bird received 0.28 mg. 16-epioestriol (8.4×10^6 c.p.m.). Urine and faeces voided subsequently to the injection were collected separately from each bird for the succeeding seven days.

C. EXPERIMENTAL AND RESULTS.

The crude urine collected from each bird for each twenty-four hour period was assayed for total radioactivity, and then subjected to enzymic hydrolysis, ether extraction and preliminary purification. The faeces from each bird were bulked and the aqueous extract thereof assayed for radioactivity. This extract was then subjected to enzymic hydrolysis, ether extraction and preliminary purification. The recoveries and distributions of radioactivity in the phenolic, acid and aqueous fractions from urine and faeces of each bird are presented in Table IX.

a. Initial purification and separation of urinary fractions.

The separate daily urinary phenolic fractions for days 1, 2 and 3 from bird 1 and for days 1 and 2 from bird 2 were subjected to initial separation and further purification by chromatography in the benzene - 55% methanol system.

TABLE IX

Recoveries and distributions of radioactivity in the urine and faeces of non-laying hens following intravenous injection of 16-epioestriol-16-C^{14}.

Collection period (days)	Radioactivity in crude urine or faeces (c.p.m.)	Distribution of radioactivity in various fractions Aqueous (c.p.m.)	Acidic (c.p.m.)	Phenolic (c.p.m.)
BIRD NO. 1				
		(a) Urine		
1	1,987,000 (29.2)*	228,700 (11.5)**	49,950 (2.4)**	1,349,000 (67.8)**
2	258,900 (3.8)*	61,310 (23.7)**	5,154 (2.0)**	141,400 (54.6)**
3	71,440 (1.1)*	17,520 (24.5)**	1,718 (2.4)**	52,660 (73.7)**
		(b) Faeces		
1-7	1,634,000 (24.0)*	75,000 (4.6)**	37,420 (2.3)**	1,241,000 (75.9)**
Total	3,951,340 (58.1)*			
BIRD NO. 2				
		(a) Urine		
1	2,132,000 (25.4)*	170,800 (8.1)**	34,370 (1.6)**	1,456,000 (68.3)**
		(b) Faeces		
1-7	2,000,000 (23.8)*	134,200 (6.7)**	26,430 (1.3)**	1,884,000 (94.02)**
Total	4,132,000 (49.2)*			

*Recovery of radioactivity expressed as percent radioactivity injected.

**Recovery of radioactivity expressed as percent radioactivity in crude urine or faeces.

Following chromatography and exposure to X-ray film the different zones of radioactivity on each chromatogram were cut out and eluted with methanol. The eluates from corresponding zones from the different chromatograms were made up to a definite volume and assayed for radioactivity. The recoveries and distribution of radioactivity from the chromatograms are presented in Table X. Radioautograms of the chromatograms for bird 1 are shown in Figure 8. The radioactive zones having R_f values of 0.06 and 0.43 were detected only on the radioautograms of chromatograms from the first day. The radioactive zones corresponding to 16-oxooestradiol-17β and 16-epioestriol appeared on all the radioautograms. Furthermore, the distribution of radioactivity between these two zones was essentially the same on each chromatogram.

Following this initial chromatographic separation, the eluates from corresponding zones from the different chromatograms were pooled.

b. <u>Purification and identification of radioactive 16-oxooestradiol-17β from urine</u>.

An aliquot of the presumptive 16-oxooestradiol-17β fraction was subjected to further purification by chromatography on a Celite gradient elution column. The peak tubes of radioactivity from the column were pooled and 200 micrograms of reference 16-oxooestradiol-17β added. The solvent was evaporated, the residue dissolved in a small amount of

TABLE X

Distributions of radioactivity following preliminary paper chromatography of the daily urinary phenolic fractions in the system benzene - 55% methanol.

Day after injection	R_f	Distribution of radioactivity			
		Bird No. 1		Bird No. 2	
		c.p.m.	percent	c.p.m.	percent
1	0.06**	not detected	not detected	27,750	2.6
	0.36+	1,063,500	86.0	900,500	84.8
	0.43**	21,200	1.7	24,800	2.3
	0.50++	151,800	12.3	108,600	10.2
2	0.35+	108,600	86.7	19,200	82.6
	0.49++	16,700	13.3	4,050	17.4
3	0.37+	41,900	88.8	Not measured	
	0.48++	5,300	11.2	Not measured	

*The distributions of radioactivity are only approximate because of some overlapping of zones.

**Not identified.

+Identified chromatographically as 16-epioestriol.

++Identified chromatographically as 16-oxooestradiol-17β.

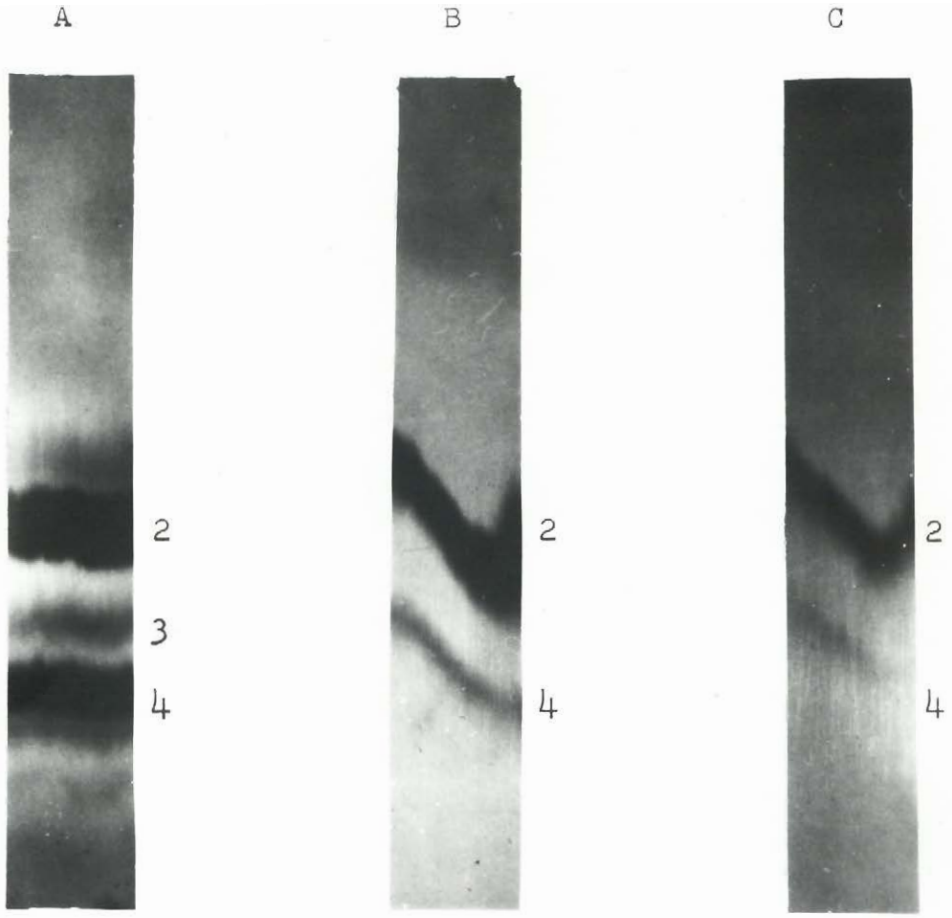


FIGURE 8. RADIOAUTOGRAMS OF CHROMATOGRAMS* OF THE PHENOLIC EXTRACTS FROM 24-HOUR URINE SAMPLES FOLLOWING INJECTION OF 16-EPIOESTRIOL-16-C^{14}.

A. Radioautogram of the first 24-hour urine extract.

B. Radioautogram of the second 24-hour urine extract.

C. Radioautogram of the third 24-hour urine extract.

NOTE: Zone 1 (not shown) was not identified; zone 2 was subsequently identified as 16-epioestriol; zone 3 was not identified; zone 4 was subsequently identified as 16-oxooestradiol-17β.

*System benzene-55% methanol.

methanol, and the material was run successively in two chromatographic systems. In each instance the bulk of the radioactivity ran with the carrier 16-oxooestradiol-17β. The pool recovered from the chromatograms was then recrystallized to constant specific activity following the addition of 18.38 mg. of reference 16-oxooestradiol-17β. Both crystals and mother liquors were analysed. The first crystallization removed a radioactive product other than 16-oxooestradiol-17β, as evidenced by the specific activity of the mother liquor. Specific activities remained essentially constant thereafter. Following the third crystallization, the crystals were acetylated and again recrystallized to constant specific activity. A summary of the recoveries and specific activities for the above purification steps is presented in Table XI.

c. Purification and identification of radioactive 16-epioestriol from urine.

The radioactive 16-epioestriol was purified in a manner similar to that described for the radioactive 16-oxooestradiol-17β. The recoveries and specific activities for the purification steps are summarised in Table XII.

d. Attempted identifications of the two minor radioactive materials from the first twenty-four hour urine samples.

These two radioactive materials together accounted for only approximately 4% of the total radioactivity recovered in the phenolic fraction. Attempts were made to

TABLE XI

A. Purification of radioactive 16-oxooestradiol-17β from the urine of non-laying hens.

Purification step	c.p.m.	µg.*	Specific Activity (c.p.m./µg.)
Pool off Celite column	101,500	---	---
Paper chromatography			
Benzene - 55% methanol	77,450	146.4	530
Toluene - 70% methanol	62,550	123.5	506

B. Crystallization of 16-oxooestradiol-17β to constant specific activity.

Purification step	c.p.m.	mg.	Specific Activity (c.p.m./mg.)
Original pool	50,300	18.38	2736
1st crystallization	26,400	14,19	1861
Mother liquor	20,250	4.72	4291
2nd crystallization	16,000	8.44	1895
Mother liquor	7,750	3.96	1956
3rd crystallization	10,450	5.68	1840
Mother liquor	5,150	2.77	1860

C. Crystallization of 16-oxooestradiol-17β diacetate to constant specific activity.

Purification step	c.p.m.	mg.	Specific Activity (c.p.m./mg.)
1st crystallization	6,550	4.59	1417
Mother liquor	1,350	0.97	1392
2nd crystallization	5,050	3.56	1419
Mother liquor	350	0.26	1347

*Estimated by Ittrich's (1958) modification of the Kober reaction.

TABLE XII

A. Purification of radioactive 16-epioestriol from the urine of non-laying hens.

Purification step	c.p.m.	µg.**	Specific activity (c.p.m./µg.)
Pool off Celite column	893,400*	---	---
Paper chromatography			
Benzene - 55% methanol	417,500	166.6	2505
Toluene - 70% methanol	347,500*	140.0	2480

B. Crystallization of 16-epioestriol to constant specific activity.

Purification step	c.p.m.	mg.	Specific activity (c.p.m./mg.)
Original pool	97,500	22.26	4379
1st crystallization	44,600	10.92	4084
Mother liquor	43,500	10.23	4253
2nd crystallization	32,500	7.89	4119
Mother liquor	8,400	1.97	4268

C. Crystallization of 16-epioestriol triacetate to constant specific activity.

Purification step	c.p.m.	mg.	Specific activity (c.p.m./mg.)
1st crystallization	16,650	5.80	2870
Mother liquor	4,300	1.48	2911
2nd crystallization	10,800	3.74	2892
Mother liquor	4,500	1.58	2853

*Aliquots taken for further purification.

**Estimated by Ittrich's (1958) modification of the Kober reaction.

identify them by chromatography. The more polar of the two zones (R_f = 0.06) had a chromatographic mobility in the benzene - 55% methanol system similar to that of oestriol. It was purified further by successive chromatography in the toluene - 70% methanol and chloroform-formamide systems. In each case the radioactivity was concentrated in a single zone, although in the second chromatography part of the radioactivity stayed at the origin. The radioactive zone from the second chromatography was eluted from the chromatogram and crystalline oestriol (30.74 mg.) was added. The total radioactivity present at this stage was 8750 c.p.m. (sp. activity 284 c.p.m./mg.). Following the first crystallization from aqueous methanol, the specific activity of the crystals dropped to 90 c.p.m. After a second crystallization the specific activity had dropped further to 46 c.p.m. It was clear, therefore, that the radioactive material contained little, if any, oestriol.

Attempts to identify the second unknown zone (R_f = 0.43) were also unsuccessful. The radioactive material was chromatographed in the benzene - 55% methanol system, along with pilot strips containing reference 16β-hydroxyoestrone and reference 17-epioestriol. The radioactive material was located on the chromatograms by radioautography whilst the reference materials were located by developing the strips with diazotised sulphanilic acid. In neither case did the radioactive spot show agreement as to position with the reference oestrogens.

D. DISCUSSION.

The results reported here show that following intravenous injection of 16-epioestriol-16-C^{14} into the fowl, the urine contained two main radioactive constituents which have been identified as 16-epioestriol and 16-oxooestradiol-17β. Two other minor constituents were not identified.

The results of the preliminary paper chromatography (Table X) showed that the distribution of radioactivity as between the 16-oxooestradiol-17β and 16-epioestriol for the twenty-four hour periods remained fairly constant, a result which indicates a continuous oxidation of 16-epioestriol to 16-oxooestradiol-17β. This is in contrast to the reduction *in vivo* of 16-oxooestradiol-17β to 16-epioestriol, since in the second twenty-four hour period following injection of 16-oxooestradiol-17β-16-C^{14} into the fowl, the amount of 16-oxooestradiol-17β in the urine was very small. These facts suggest a high efficiency of conversion of 16-oxooestradiol-17β to 16-epioestriol compared to the reverse reaction.

Furthermore, the fact that oestriol could not be detected in the urine following injection of either 16-oxooestradiol-17β or 16-epioestriol suggests that the fowl may lack a 16α-hydroxysteroid dehydrogenase system. On the other hand, since it has also been demonstrated that the fowl is capable of converting injected oestriol-16-C^{14} into 16-oxooestradiol-17β and 16-epioestriol (MacRae *et al.* 1960),

it is conceivable that *in vivo* in the fowl, the equilibrium,

oestriol ⟶ 16-oxooestradiol-17β ⇌ 16-epioestriol

is such that it is displaced very much to the right. At present we are inclined to the view that the results are most simply explicable on the theory that 16-epioestriol is formed from 16-oxooestradiol-17β at the expense of oestriol. However, if this is so, then the formation of oestriol as a urinary conversion product of oestrone-16-C^{14} (Ainsworth and Common, 1962) may be suspect, since if the fowl does indeed lack a 16α-hydroxysteroid dehydrogenase system, any formation of oestriol must have taken place via a pathway which does not involve the formation of the intermediate α-ketols.

The foregoing facts are in contrast to the conditions which apply in the human species, since both *in vivo* (Levitz *et al.* 1960) and *in vitro* (Breuer *et al.* 1958) experiments have shown that oestriol is the predominant conversion product of 16-oxooestradiol-17β. Further, it has been reported that the oxidation of 16-epioestriol to 16-oxo-oestradiol-17β can take place in the human following injection of 16-oxooestradiol-17β-16-C^{14} (Levitz *et al.* 1960). A further point of interest in the results now reported is the high proportion of radioactivity which remained in the aqueous phase following extraction of the urine with ether in the second and third days following injection, as compared with the first day (see Table IX). This fact suggests the

possibility that 16-epioestriol may undergo further conversion to products which are water-soluble. Furthermore, a much higher proportion of the total radioactivity excreted was present in the faeces than had been observed in previous experiments with labelled oestrogens (Ainsworth and Common, 1962; Table III, above).

CHAPTER V

SUMMARY - PART 1

1. Oestrone and equol have been isolated in crystalline form for the first time from extracts of large amounts of avian urine (128 days). The identity of each compound has been established by comparison of its infrared spectrum with that of the appropriate reference compound. Attempts to isolate oestradiol-17β and oestriol from the same source were unsuccessful.

2. 16-Oxooestradiol-17β-16-C^{14} was injected intravenously into non-laying hens furnished with exteriorized recti. The urine and faeces for succeeding days were collected separately. Oestrogen extracts were prepared using enzymic hydrolysis. Analyses of the urinary phenolic fraction showed the presence of two major radioactive materials and one minor radioactive material. The two major radioactive materials were identified as 16-oxooestradiol-17β and 16-epioestriol. The minor radioactive material was not identified, but it contained little, if any, radioactive oestriol. The faecal phenolic fraction contained a radioactive material identified as 16-epioestriol.

3. 16-Epioestriol was injected into two non-laying hens furnished with exteriorized ureters. The urine and faeces for succeeding days were collected separately, and oestrogen extracts thereof were prepared by use of enzymic hydrolysis.

The urine contained two radioactive products which were identified as 16-epioestriol and 16-oxooestradiol-17β. Two minor radioactive products were not identified but neither was oestriol or 16-epioestriol or 16β-hydroxyoestrone.

4. A summary of recoveries and distributions of the radioactivity in both experiments has been presented along with criteria for the radiochemical purities of the isolated steroids.

5. The results have been discussed in relation to the view that 16-epioestriol is an important oestrogen conversion product in the fowl.

PART II

STUDIES ON THE QUANTITATIVE ESTIMATION OF NATURALLY OCCURRING OESTROGENS IN THE URINE OF THE DOMESTIC FOWL

CHAPTER I

REVIEW OF THE LITERATURE

A. METHODS FOR THE QUANTITATIVE ESTIMATION OF OESTROGENS.

Bauld and Greenway (1957), Greene and Touchstone (1959), O'Donnell and Preedy (1961), Diczfalusy and Lauritzen (1961) and Preedy (1962) have discussed at length all the important methods that have been used for the determination of oestrogens in human urine and other body fluids and tissues. Consequently, this review will be restricted to a consideration of the more important methods.

The following types of methods have been used for the estimation of oestrogens.

1. BIOLOGICAL METHODS.

With these methods the oestrogenic activity of a sample has been measured by studying the biological effect on animals, e.g., the effect on the vaginal smear (Allen and Doisy, 1923) or the effect on uterine growth in immature rats (Dorfman et al. 1935). Numerous modifications of these two methods exist. The subject of bioassay of oestrogens has been reviewed recently by Emmens (1962) and will, therefore, not be discussed further here. It might be remarked, however, that these methods are by far the most sensitive in that amounts as small as 10^{-5} micrograms of oestradiol-17β or oestrone can be assayed.

2. PHYSICAL METHODS.

None of the earlier physical methods have been widely used since they are mostly not sensitive enough and in some instances extreme purity of the sample is necessary, e.g., ultraviolet and infrared absorption spectroscopy. The necessary extensive purification cannot be achieved when working with biological material at low oestrogen concentration.

Two types of gas chromatographic method have been developed recently for the estimation of oestrogens. Wotiz and Martin (1962) and Luukkainen et al. (1962) have incorporated gas chromatography into methods for the estimation of urinary oestrogens in late pregnancy. These methods involved the synthesis of the acetates or of the trimethylsilyl ethers of the oestrogens prior to chromatographic separation. The sensitivity of the former method (based upon the pure compound) was approximately 0.15 micrograms of oestrogen whilst in the latter 0.03-0.05 micrograms of the trimethylsilyl ether derivative of the oestrogen could be detected. Fishman and Brown (1962) have compared the values for oestrone, oestradiol-17β and oestriol from human pregnancy urine as obtained by gas chromatography with those obtained by the method of Brown (1955). They found that the results obtained were comparable, even though the purification of the extracts for gas chromatography were minimal. They stated, however,

that in extracts with less oestrogen, larger portions of extract would have to be chomatographed and more extensive purification would be necessary. More recently, Wotiz (1963) has modified his earlier procedure (Wotiz, 1962) to include the separation and estimation of 16-epioestriol in oestrogen extracts of human pregnancy urine.

3. ENZYMIC METHODS.

Two entirely different enzymic methods for the determination of steroid hormones, including oestrogens, have been developed. Gordon and Villee (1956) have described a method based on the influence of oestrogens on an NAD-dependent isocitric dehydrogenase system in human placental extracts. Hurlock and Talalay (1957, 1958) developed a method based on the interconversion of certain hydroxy- and oxosteroids by NAD-linked hydroxysteroid dehydrogenases prepared from the organism Pseudomonas testosteroni (Marcus and Talalay, 1956). In the latter method a dehydrogenase could be used which acted on both 3β and 17β hydroxyl groups and could, therefore, be used to measure oestrone, oestradiol-17β and oestriol. However, the enzyme cannot distinguish between the three oestrogens, or between oestrogens and other steroids having hydroxyl groups in the same position.

4. RADIOCHEMICAL METHODS.

Both C^{14}-labelled and H^{3}-labelled oestrogens have been used for the estimation of oestrone, oestradiol-17β

and oestriol. Isotope dilution and reverse isotope dilution techniques have been used for the establishment of the specificity of the methods of Brown (1955; 1957b; Gallagher et al. 1958), and the method of Preedy and Aitken (1961b) for the determination of oestrogens in urine and plasma.

Svendsen (1960) has described a method for the determination of oestrone and oestradiol-17β in plasma based on the double isotope derivative principle. The method included extraction of the plasma with chloroform, purification, esterification of oestrone and oestradiol-17β by S^{35}-P-iodobenzene sulphonyl chloride, addition of known amounts of I^{131}-P-iodobenzene sulphonyl chloride esters of oestrone and oestradiol-17β, separation of the esters by paper chromatography and determination of radioactivities.

Recently, Slaunwhite and Neely (1963) have introduced a method for analysis of oestrogens based upon bromination of the oestrogens with Br^{82}. Oestrone, oestradiol-17β and oestriol undergo bromination in the 2 and 4 positions. Oestrogen extracts were dissolved in glacial acetic acid and then subjected to bromination. The brominated oestrogens were extracted with chloroform, the chloroform evaporated, and the residue dissolved in benzene and transferred to an alumina column along with carrier dibromoestrogens. The oestrogens were eluted from the column by gradient elution with ethanol in benzene (2 ml. fractions collected). Following chromatography, the radioactivity and absorption at 291 mμ were assayed

for each fraction. When a peak of radioactivity coincided with an absorption peak, 50 mg. of appropriate carrier was added and the whole crystallized several times to constant specific activity. The quantity of oestrogens was calculated on the basis of the radioactivity yielded by 100 millimicrograms of authentic oestrone, correcting for decay when necessary.

The method was shown to be very sensitive (1.0 millimicrograms), reasonably accurate (80% recovery from urine, 95% recovery from plasma at the 10 millimicrogram level), quite precise (± 3%) and highly specific. The specificity was assured by gradient elution chromatography and recrystallization to constant specific activity.

5. CHEMICAL METHODS.

The most important methods for the determination of oestrogens are the fluorimetric and colorimetric methods.

a. Fluorimetric methods.

Oestrogens exhibit an intense fluorescence when heated with either phosphoric or sulphuric acid. The application of sulphuric acid fluorescence to the estimation of oestrogens was introduced by Jailer (1947, 1948) and modified by Bates and Cohen (1950a, b). Finkelstein et al. (1947) and Finkelstein (1952) recommended the use of phosphoric acid. They claimed that the procedure, although less sensitive than that involving sulphuric acid, was subject to less interference. The many problems involved in

fluorimetry have been reviewed adequately by Bauld and Greenway (1957) and will not be discussed here.

The sensitivity of fluorimetric estimations is very high and, according to Preedy and Aitken (1961a), 0.005 micrograms of oestrone can be measured with accuracy. Optimal conditions for the measurement of pure oestrogens by sulphuric acid fluorescence have been investigated by Slaunwhite et al. (1953), Goldzieher (1953), Aitken and Preedy (1953) and Bauld et al. (1960).

However, extracts of biological material frequently contain non-oestrogenic materials which also give fluorescence with sulphuric acid and consequent interference with the specificity of the method. Optimal conditions for estimating oestrogens in urine extracts by sulphuric acid fluorescence after column partition chromatography have been presented by Preedy and Aitken (1961a) and have been incorporated into a method for the determination of oestrone, oestradiol-17β and oestriol in urine and plasma (Preedy and Aitken, 1961b).

b. Colorimetric methods.

The Kober (1931) reaction, as modified by Bauld (1954), is the best known and has been used extensively for the estimation of oestrogens. It has been incorporated into the methods of Brown (1955) and of Bauld (1956) for the determination of oestrogens in human urine. The reaction depends on heating the oestrogen with aqueous sulphuric acid, cooling, adding water and reheating. A pink colour

is developed which is measured spectrophotometrically at 480, 516 and 552 mμ .

The Kober reaction, although exhibiting a considerable degree of specificity, is not of sufficient specificity to enable oestrogens to be determined in the presence of large amounts of interfering material. In both the Brown (1955) and Bauld (1956) methods the Allen (1950) spectrophotometric correction has to be applied to correct for the colour due to interfering chromogens produced during the reaction. Furthermore, the Kober reaction as such is much less sensitive than fluorescence and cannot be applied with any degree of accuracy to the estimation of oestrogens in blood and plasma (O'Donnell and Preedy, 1961).

Nocke (1961a) studied the influence of various reaction conditions on the colour production by five naturally occurring oestrogens in the Kober reaction. In addition to the 'classical' oestrogens, oestradiol-17α and 16-epioestriol were studied. It was found that the optimal conditions for the classical oestrogens in the first stage of the Kober reaction were the same as those described by Brown (1955). However, for complete conversion of the yellow colour into pink it was found that lower sulphuric acid concentrations than those reported by Brown (1955) were necessary for optimal development of the pink colour. A further point of interest was the behaviour of oestradiol-17α in the colour reaction. This compound underwent the first stage of the reaction at room temperature within a

few minutes and it very readily gave an intense Kober colour, which was particularly susceptible to variation in the reaction conditions.

Ittrich (1958) has described a method of chemical oestrogen determination based on a specific extraction of the Kober colour complex with a solution of 2% p-nitrophenol and 1% ethanol in chloroform. The spectrophotometric examination of the extracted colour complex has shown that in addition to an increase in colour intensity, there was a shift in the maximum absorption to a longer wavelength, as compared with the original colour reaction. Besides showing a higher sensitivity, the Kober reaction carried out in this way has been found to be considerably more specific than earlier modifications (Ittrich, 1958; Salokangas and Bulbrook, 1961).

In addition to its favourable properties with regard to spectrophotometric measurements, the extracted colour complex produced an intense yellowish-green fluorescence. By use of the conditions described by Ittrich (1958), it was possible to attain a sensitivity comparable to that of sulphuric acid fluorescence procedures. The extraction of the Kober complex by organic solvents was further modified by using a mixture of 2% p-nitrophenol and 1% ethanol in ethylene tetrabromide (Ittrich, 1960). The use of this solvent gave optimal conditions for fluorimetry. Furthermore, Salokangas and Bulbroom (1961) recommended the use of 2% p-nitrophenol

in tetrachlorethane when extracting the Kober colour complex for spectrophotometric determination.

Stoa and Thorsen (1962) have investigated the sensitivity, specificity and precision of the Ittrich (1960) extraction procedure in combination with spectrofluorimetric determinations. They concluded that the degree of specificity was considerably higher in spectofluorimetry than when spectrophotometry was used. Furthermore, it was shown that determinations of oestriol in urinary extracts prepared by a comparatively simple fractionating technique, gave satisfactory specificity and precision.

The usefulness of Ittrich's (1958, 1960) methods has been investigated by several other authors (Breuer and Gertz, 1960; Lutzmann and Wurterle, 1960; Salokangas and Bulbrook, 1961). From these investigations it can be concluded that although the extraction principle of Ittrich may be advantageous in certain respects as compared with the conventional Kober reaction, it does not really represent any fundamental advance. However, the reservation was made in most cases that the method would possibly prove to be more advantageous when adopted for fluorimetry.

B. GENERAL PROCEDURES FOR THE EXTRACTION, PURIFICATION AND ESTIMATION OF OESTROGENS PRESENT IN HUMAN URINE.

Most of the procedures relate to the estimation of the 'classical' oestrogens, viz., oestrone, oestradiol-17β and oestriol, and in this section reference will be made only to those methods which have been adequately tested and assessed.

The general principles involved in the estimation of oestrogens in urine are:- hydrolysis, extraction, purification, separation and quantitative estimation. With the exception of quantitative determination, the principles have been discussed previously (Part I, Chapter I) and will not be discussed further.

Since 1955 three methods for the estimation of the 'classical' oestrogens in human urine, involving the above principles, have been devised and adequately evaluated. These are the methods of Brown (1955), later modified by Brown et al. (1957b), of Bauld (1956) and of Preedy and Aitken (1961b).

The best known of these methods is that of Brown (1955). This method involved acid hydrolysis, ether extraction, solvent partition, and a phase-change purification step which involved methylation followed by separation of the oestrogen methyl ethers on partially deactivated alumina columns. Finally, the amounts of the three compounds were measured by a modified Kober (1931) reaction. The Allen (1950) spectrophotometric correction

was applied to the spectrophotometric readings to eliminate the contribution of non-specific chromogens. The modification introduced by Brown et al. (1957b) involved the incorporation of an additional purification step. This consisted of a saponification with 1.0 N NaOH prior to methylation of the oestrogen fractions. This additional step, first suggested by Bauld (1956), enhanced the specificity of the method considerably. The whole method has been thoroughly investigated as to its reliability (Brown et al. 1957a; Breuer et al. 1957; Gallagher et al. 1958; Brown and Blair, 1960; Fishman and Brown, 1962).

A further modification has been introduced by Brown and Blair (1960) to permit measurement of the small amounts of oestrone in the urine of oophorectomised and adrenalectomised patients. In this method, a Girard separation has been included prior to solvent partition. The rest of the procedure was the same as that described by Brown et al. (1957b). However, it was stated that the oestrone content of these low-titre urines was overestimated by 0.9 micrograms per twenty-four hours whenever the procedure of Brown et al. (1957b) was used for analysis.

The method of Bauld (1956) differs significantly from that of Brown et al. (1957b) only in the absence of a methylation of the oestrogens and in the type of chromatography used. The method of chromatography involved separation of the oestrogens on two Celite partition columns (one for oestrone and oestradiol-17β, one for

oestriol), followed by the Kober reaction, with the Allen correction.

The method of Preedy and Aitken (1961b) differs from the two methods mentioned previously in that after hydrolysis and extraction of the steroids with ether, the ether was evaporated and the residue subjected to partition between toluene and 1.0 N NaOH. The aqueous phase was then adjusted to pH 9.0 and a further ether extraction carried out. The dried ether extract was then submitted to column partition chromatography. Fractions of eluate were collected, and the oestrogen content of each determined by sulphuric acid fluorescence.

The various advantages and disadvantages of these three methods have been adequately discussed by Preedy (1962) and will not be included here.

The rapidly increasing number of newly discovered oestrogens has already been referred to in Part I, Chapter I. In this connection, Givner et al. (1960a, b) have described a method for the quantitative fractionation and determination of mixtures of 2-methoxyoestrone, oestrone, ring D α-ketolic oestrogens, oestradiol-17β, 16-epioestriol and oestriol in human urine. The technique involved enzymic hydrolysis of the urine, ether extraction, separation of the individual oestrogens by column partition chromatography on Celite and colorimetric estimation of the individual fractions by the Kober reaction.

Recently, the method of Brown et al. (1957b) has been modified by Nocke and Breuer (1963) to include the separation and estimation of 16-epioestriol in the form of its acetonide. Following solvent partition of the ether extract between benzene/hexane and water, the aqueous phase (which contained 16-epioestriol and oestriol) was saponified with 1.0 N NaOH, brought to pH 9.3 and extracted with ether. The residue which remained after evaporation of the ether was then subjected to acetonide formation using the procedure of Nocke (1961b). Following evaporation of acetone, the residue was partitioned between chloroform and 0.4N NaOH. The chloroform extract contained the 16-epioestriol acetonide, whereas the oestriol, being strongly phenolic, remained in the aqueous alkaline phase. The oestriol fraction was then methylated and chromatographed on alumina by Brown's method (1957b). The 16-epioestriol acetonide fraction was freed from chloroform, dissolved in benzene and then chromatographed on alumina. Following elution of the oestriol and 16-epioestriol derivatives from the respective columns, the amount present was estimated by the Kober reaction. Oestrone and oestradiol-17β were purified and estimated in the usual way (Brown, 1957b).

C. THE ESTIMATION OF OESTROGENS IN THE URINE OF DOMESTIC ANIMALS.

In recent years several techniques, based on the methods of Brown (1955, 1957b) or of Bauld (1956), have been

developed for extraction, separation and estimation of urinary oestrogens from domestic animals.

El-Attar and Turner (1957) studied the urinary and faecal oestrogen excretion of cows during different stages of pregnancy. Hydrolysis of the urine samples was carried out by the addition of 5% (v/v) sulphuric acid followed by refluxing for 30 minutes at 80°C. The cooled solutions were extracted with ether and the ether extract then submitted to a purification procedure similar to that described by Brown (1955). The ether was evaporated and the residue was partitioned between toluene and 1.0 N NaOH. The organic phase, following extraction four times with 1.0 N NaOH, was discarded and the combined aqueous phases were brought to pH 9.3-9.5 and extracted with ether. This extract contained the phenolic fraction. The phenolic fraction was then separated into ketonic and non-ketonic fractions by use of Girard's reagent T. Each fraction was then estimated using a sulphuric acid fluorescence technique. Faecal samples were similarly processed following extraction of the oestrogens with 95% ethanol.

Their results indicated that urinary and faecal oestrogen levels remained low until about 170 days of pregnancy and then began to increase markedly. The average total (urine and faeces) oestrogen excretion per twenty-four hours was 4.49 mg. (calculated as oestradiol) in four cows pregnant 91-109 days, 5.56 mg. in five cows pregnant 120-170 days, 19.11 mg. in six cows pregnant 185-234 days,

and 20.13 mg. in five cows pregnant 248-273 days. In fourteen cows the faecal oestrogen excretion was at a higher level than urinary oestrogen, while in six cows, pregnant more than six months, the urinary oestrogen exceeded the faecal oestrogen. The major oestrogen in cows' urine and faeces was in the non-ketonic phenolic fraction, calculated as oestradiol.

Velle (1958a, 1958b) used a slight modification of the method of Brown et al. (1957b), in which the urine was hydrolysed by heating with 6% (v/v) hydrochloric acid for one hour in boiling water, for the estimation of urinary oestrogens from the pregnant cow. He showed that oestrone and oestradiol-17α were quantitatively the most important urinary oestrogens excreted during pregnancy. An increase in urinary oestrogen excretion was observed from about the 80th day after conception. Further studies on the urinary oestrogen excretion by the newborn calf (Velle, 1958f) showed that during the first two weeks of life, the average total daily excretion in the males and females, respectively, were calculated to be 0.23 and 0.24 mg. of oestrone and 3.3 and 2.3 mg. of oestradiol-17α. Hydrolysis of urine was carried out using 3% (v/v) hydrochloric acid and a heating time of 30 minutes (in boiling water) to obtain maximal yield of oestradiol-17α. The levels fell off rapidly after the first four days of life but significant amounts of both oestrogens could be found throughout the whole period investigated.

Velle (1958g) has made quantitative studies on the urinary excretion of oestrogens by the boar using the method of Brown (1955). The average values for ten, adult, fertile boars were 1.60 mg. oestrone and 0.86 mg. oestradiol-17β, calculated per litre of urine. Great variability was observed both in each animal and between the animals.

Velle (1958h, 1959) has provided evidence that oestrone is the principal oestrogen in the urine of the pregnant and non-pregnant sow. The amounts of oestradiol-17β and oestriol were insignificant during the oestrus cycle. Lunaas (1962) subsequently investigated the urinary oestrone excretion in the sow during the oestrous cycle and early pregnancy. The patterns of urinary oestrone levels were characterised by a rise of the levels associated with oestrus to a well-defined peak followed by a sudden decrease, usually to the lowest levels observed. Similar observations on oestrone excretion during oestrus have been reported by Raeside (1961).

Lunaas (1962) found that the levels of oestrone after conception were not appreciably different from those during the oestrus cycle until the end of the third week, after which time the excretion of oestrone increased. The maximal excretions of oestrone in the three sows studied were observed on days 24, 25 and 27 after conception. Thereafter, the oestrone excretion dropped rapidly. Consistent fluctuations during the oestrus cycle, or after conception, of urinary oestradiol fractions were not

recorded. These results supported the previous findings of Velle (1958h) that the levels of urinary oestrone after conception do not differ appreciably from those during the oestrus cycle until the end of the third week, after which the levels rise to attain a peak at the end of the fourth week.

Recently, Raeside (1963) has developed a procedure for the chemical determination of oestrone and oestradiol-17β in the urine of non-pregnant sows. The procedure involved acid hydrolysis; ether extraction; purification by adsorption chromatography on alumina and saponification; separation of oestrone and oestradiol-17β by column partition chromatography; further purification of the oestrone fraction with Girard's reagent; colorimetric measurement with the Kober reaction, followed by extraction of the Kober colour complex with 2% p-nitrophenol in tetrachlorethane. The reliability of the method in terms of its accuracy, precision, sensitivity and specificity was also evaluated.

Attempts to measure urinary or faecal oestrogen levels in ewes have not been very successful due to the small amounts of oestrogen present, even in late pregnancy (Velle, 1958h). However, Velle (1958h) did obtain some evidence for an increase of both urinary oestrone and oestradiol-17α in late pregnancy. Wright (1962) used the method of Brown et al. (1957b) and the Ittrich (1958) modification of the Kober reaction for the determination of oestrogens in the faeces of sheep. It was found, however,

that large amounts of unknown substances interfere in the development of the Kober colour. This interference may be reduced to some extent by a careful choice of diet, but the problems associated with the relatively large amounts of interfering substances in faecal extracts are far from solution.

CHAPTER II

MATERIALS AND METHODS

A. GENERAL MATERIALS AND APPARATUS.

1. REFERENCE STANDARDS.

Crystalline oestrone-3-methyl ether, crystalline oestradiol-17β-3-methyl ether, crystalline oestriol-3-methyl ether and crystalline oestriol were obtained from Sigma Chemical Company, 3500 DeKalb St., St. Louis 18, Missouri, U.S.A.

Crystalline oestradiol-17β and crystalline oestrone were obtained from Steroid Laboratories Ltd., Box 247, Montreal, Canada.

Crystalline 16-epioestriol was presented by Dr. D.S. Layne, Worcester Foundation for Experimental Biology, Shrewsbury, Mass., U.S.A.

2. REAGENTS.

All reagents were of analytical grade unless otherwise stated. All solvents used in the Brown (1955) procedure were purified as specified by Diczfalusy and Westman (1956).

Solvents for use in thin-layer chromatography were purified as described by Hertelendy and Common (1963).

The reagents used in the Ittrich (1958) modification of the Kober reaction were as follows:-

Concentrated sulphuric acid ('Analar') and quinol (laboratory reagent); obtained from British Drug Houses Ltd., Toronto, Ontario.

1,1,2,2-Tetrachlorethane (D. 1.596/20°C) (Eastman Organic Chemicals), 'spectranalysed' grade methanol and p-nitrophenol; obtained from the Fisher Scientific Company, Montreal, P.Q.

3. CHROMATOGRAPHIC MATERIALS.

Alumina, 'Woelm' neutral, activity grade 1, obtained from Alupharm Chemicals, P.O. Box 755, New Orleans, La., U.S.A.

Dowex 2-X8, chloride form (35-41% moisture, 50-100 mesh), obtained from Dow Chemical of Canada Ltd., Sarnia, Ontario.

Silica gel G (Merck, Darmstadt) for use in thin layer chromatography, obtained from Canadian Laboratory Supplies, Montreal, Quebec.

4. APPARATUS.

Absorption chromatography with alumina was carried out using 14 x 130 mm. glass columns fitted with a sintered glass disc.

Ion-exchange chromatography with Dowex-2 resin was carried out using apparatus similar to that described by Engel et al. (1961).

The Desaga outfit for thin-layer chromatography was

purchased from Brinkmann Instruments Inc., Great Neck, New York, N.Y., U.S.A. 'Kober Tubes' (Pyrex glass; 150 x 16 mm.) with standard TS 13 ground glass stoppers were obtained from Fisher Scientific Company, Montreal, P.Q.

Optical densities were measured with a Beckman Model DU spectrophotometer obtained from Beckman Instruments Inc., Fullerton, California, U.S.A.

B. METHODS.

1. COLLECTION AND STORAGE OF URINE SAMPLES.

Urine was collected from laying and non-laying hens which had been surgically modified so as to provide them with exteriorised ureters. The technique for exteriorization of the ureters was essentially that used by Dixon and Wilkinson (1957) with certain minor modifications incorporated by the present author. This technique is described in detail in the Appendix.

Urine was collected twice daily and was processed immediately a twenty-four hour sample had been collected, or kept at -20°C in the deep freeze until required.

2. PREPARATION OF URINE FOR HYDROLYSIS.

Each twenty-four hour urine sample was first filtered through a Büchner funnel with the aid of suction to separate the solid material. The latter was then ground up with 50 ml. distilled water and again filtered. This filtrate was added to the first filtrate and the volume made up to 500 ml. with distilled water. The urine was

then processed as outlined below (Figure 9).

3. THE METHOD OF BROWN (1955) AND ITS MODIFICATIONS.

The method used for extraction and purification of oestrogens from the urine of the domestic fowl was essentially that described by Brown (1955) with the slight modifications proposed by Diczfalusy and Westman (1956). The steps involved are presented in Figure 9.

The alumina was deactivated by the addition of between 9-10% water. However, the degree of deactivation can be stated only approximately for the method. The final adjustment must be checked with each batch of alumina by the standardization procedure of Brown (1955).

4. COLOUR REACTIONS.

Throughout this work estimations of oestrogens have been made using the Ittrich (1958) modification of the Kober reaction with selective extraction of the colour produced with 2% p. nitrophenol and 1% ethanol in tetrachlorethane as recommended by Salokangas and Bulbrook (1961).

Detection of oestrogens on thin-layer chromatoplates was performed by spraying the plates with 2% sulphuric acid in 50% ethanol followed by heating the plates at 100°C for 15 minutes to develop the colour.

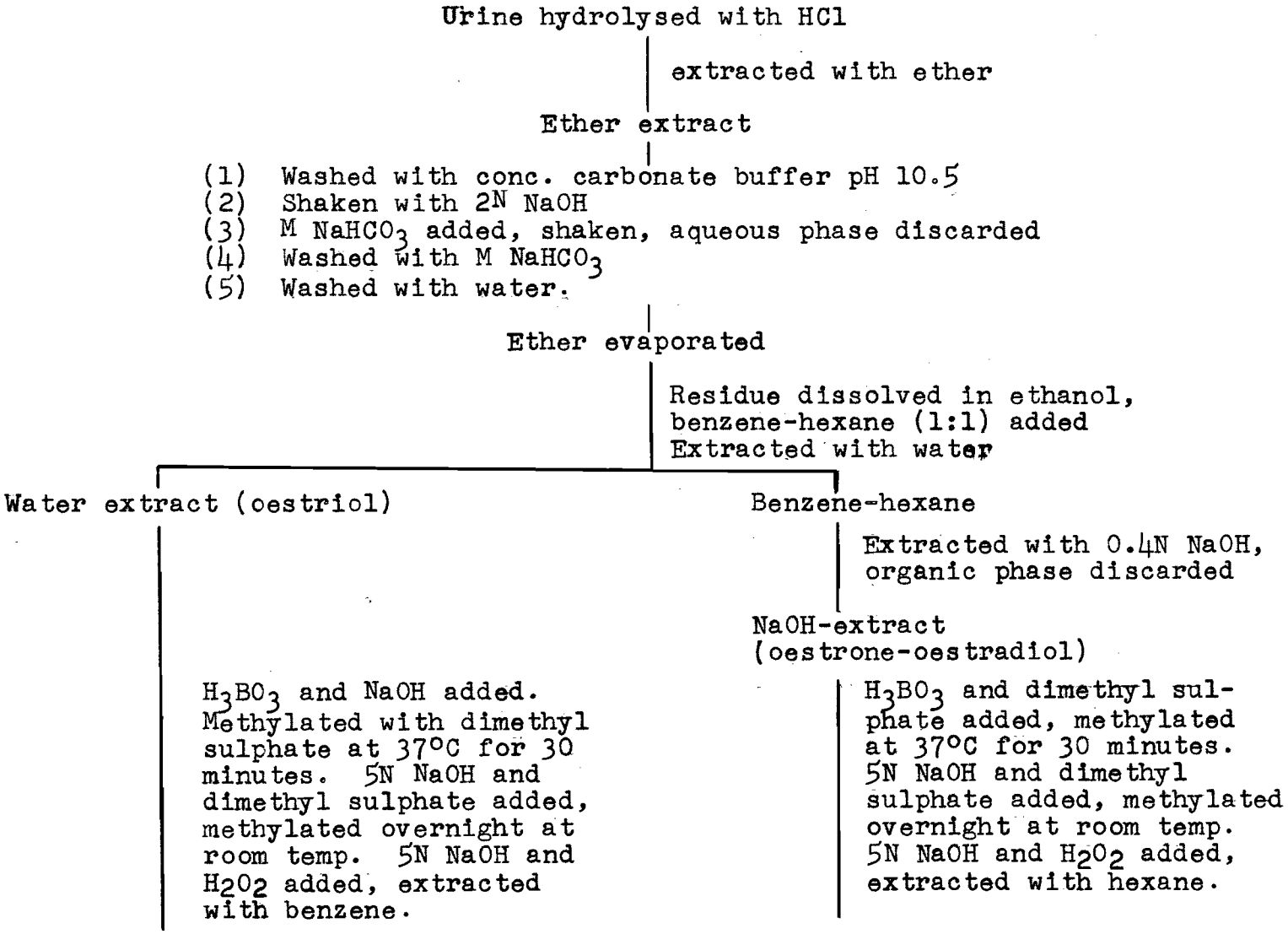


FIGURE 9. FLOW SHEET FOR THE SEPARATION AND ESTIMATION OF OESTRONE, OESTRADIOL AND OESTRIOL FROM AVIAN URINE BY THE METHOD OF BROWN (1955). (CONTINUED ON FOLLOWING PAGE).

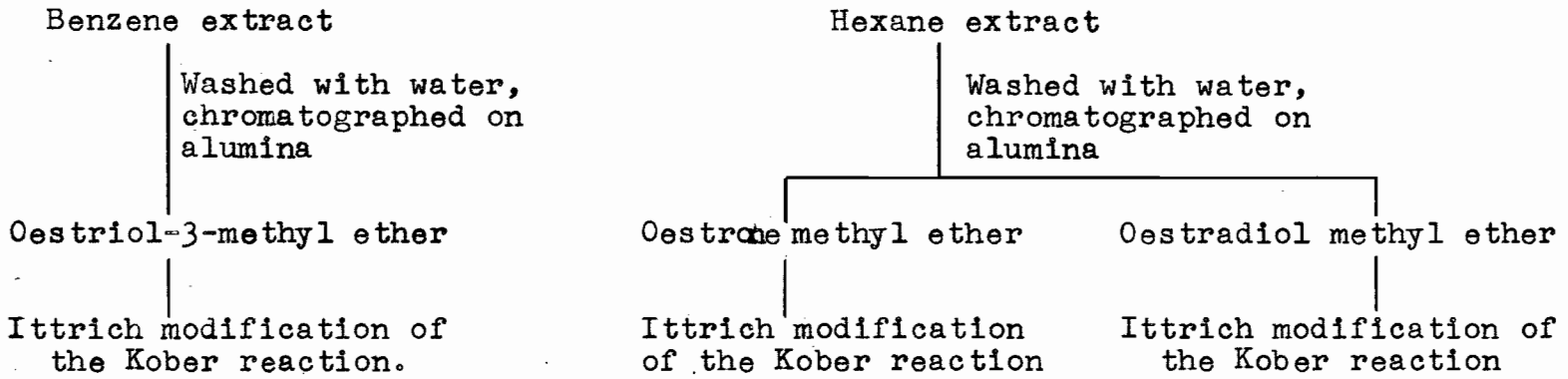


FIGURE 9. FLOW SHEET FOR THE SEPARATION AND ESTIMATION OF OESTRONE, OESTRADIOL AND OESTRIOL FROM AVIAN URINE BY THE METHOD OF BROWN (1955).

a. Preparation of reagents for the Kober reaction.

Steroids. Standard solutions of pure crystalline oestrogens and their 3-monomethyl ethers were prepared in spectranalysed grade methanol with a concentration of 10 micrograms per ml. and stored at 4°C.

Colour reagents. An aqueous solution of 65% sulphuric acid was prepared by the addition of 130 ml. of concentrated sulphuric acid to 70 ml. of water, the mixture being cooled in an ice bath. A solution of 2% (w/v) hydroquinone in ethanol was also prepared. Both solutions were stored in amber bottles at room temperature in the dark.

2% p-Nitrophenol solution was prepared by dissolving 2 g. p-nitrophenol and 1 ml. ethanol in 60 ml. 1, 1, 2, 2-tetrachlorethane. The solution was then cooled and made up to 100 ml. with tetrachlorethane. p-Nitrophenol was purified by recrystallization from benzene. This solution was stored in amber bottles at 4°C.

b. Kober reaction.

Standard solutions of oestrogens or purified oestrogen extracts from urine were evaporated in Kober tubes with 1 ml. of 2% hydroquinone in ethanol by heating in a water bath in an atmosphere of nitrogen. 1.2 ml. of 65% sulphuric acid was then added to the dry residue and the colour was developed by heating the tubes with stoppers in place in a boiling water bath for 40 minutes. The tubes were shaken twelve times under identical conditions after

2 minutes and 5 minutes of heating. After cooling the solutions in a bath of ice-water for 5 minutes, a layer of 1.5 ml. of distilled water was added to each tube. The tubes were cooled for a further 3 minutes and then mixed by rotation. After a further 3 minutes of cooling, 4 ml. of cooled p-nitrophenol solution was added and the stoppered tubes cooled again for 3 minutes. Each tube was then shaken vigorously for 20 seconds (150 times) and centrifuged for 4 minutes at a speed of 3000 r.p.m. at 0°C. The upper layer and impurities were then sucked away by use of a water pump.

c. Spectrophotometry.

Extinction (log I_o/I) was measured in a Beckman DU spectrophotometer against similarly treated reagent blanks in 10 mm. glass cells. The readings were corrected for unspecific background colour by applying the equation of Allen (1950). The extinction was measured at 505, 538, 571 mμ in the case of the free oestrogens and at 507, 540 and 573 mμ in the case of the oestrogen methyl ethers. The corrected extinction was calculated from the following formulae:-

$$E_{corr} = E_{538} - 0.5(E_{505} + E_{571})$$

or $$E_{corr} = E_{540} - 0.5(E_{507} + E_{573}).$$

CHAPTER III

A STUDY ON THE USE OF ION EXCHANGE CHROMATOGRAPHY FOR PURIFICATION OF OESTROGENS

A. INTRODUCTION.

At the present time there is a serious lack of information on the quantitative aspects of the excretion of oestrogens by the domestic fowl.

With regard to the laying hen, MacRae (1960) experienced great difficulty in attempts to arrive at quantitative results from droppings using the procedure of Bauld (1956). These difficulties arose mainly from the fact that it was not possible to attain a degree of purification of the extracts that would permit quantitative application of the Kober reaction. The main reason for this was probably due to the circumstance that it was not practicable to include the purification step involving partition chromatography of the purified oestrogen extracts on a Celite column. Furthermore, the extracts of the droppings contained a far higher proportion of interfering chromogens than was normally present in human urine. From this, it was clear that the first thing to do was to try to obtain urinary oestrogen values, since urine does not yield nearly as much interfering material in the extracts.

During the summer of 1961 the author studied the applicability of the procedure of Bauld (1956) (with the exception of the partition chromatography), and the Ittrich (1958) modification of the Kober reaction, for the determination of oestrogens in hen's urine. The results suggested that there was essentially no difference between the amounts of total Kober positive oestrogen (as thus determined) excreted by the laying hen and the non-laying hen. The range of values for seventy-five twenty-four hour urine samples from two non-laying hens and two laying hens lay within the range 2-12 micrograms oestrogen per twenty-four hours.

However, although these results provided a tentative preliminary measure of the order of magnitude of urinary oestrogen excretion, the values must be treated with considerable reserve. The reliability criteria for the analytical procedure used were practically unknown and were, in the author's opinion, almost certainly inadequate to permit of any firm statement as to the amounts of oestrogen excreted.

In view of the inadequacy, from the quantitative standpoint, of the techniques used hitherto, it was decided to explore the use of ion-exchange chromatography in conjunction with Bauld's (1956) procedure, for the estimation of total Kober positive material in urine extracts. The purpose of the ion exchange chromatography was to serve as a final purification step for the purified urinary extracts

prior to estimation of the Kober positive material by the Ittrich (1958) modification of the Kober reaction.

In this connection, a procedure involving the use of ion-exchange chromatography has been developed by Engel et al. (1961). It was designed to estimate the urinary conversion products following the administration of oestradiol-17β-16-C^{14} to human subjects. It was thought that it might be possible to adapt this method to the estimation of total Kober positive material and thus arrive at a reasonably rapid and effective quantitative measurement of total urinary oestrogen excretion.

B. METHOD

1. PREPARATION OF THE ION-EXCHANGE COLUMN AND SEPARATION OF THE OESTROGEN FRACTION.

The preparation of the column was carried out exactly as described by Engel et al. (1961). Following elution of the oestrogen containing fraction with 200 ml. 5N acetic acid in methanol, the solvent was evaporated under pressure, methanol being used to remove the last traces of acetic acid. The amount of oestrogen was then determined using the Ittrich (1958) modification of the Kober reaction.

C. RESULTS AND DISCUSSION.

1. RECOVERY OF REFERENCE OESTROGEN COMPOUNDS FROM RESIN COLUMNS.

Six 10 g. columns were prepared as outlined above. To each column was added 10 micrograms oestrone in methanol. Each column was then eluted with methanol (70 ml.) and then with 5N acetic acid in methanol (200 ml.). The latter fractions were evaporated and the amount of oestrogen estimated by the Ittrich (1958) modification of the Kober reaction. The recovery of added oestrone from the columns was of the order of 50%. At this stage it was suspected that some of the oestrogen was being eluted in the methanol fraction. Accordingly, six more columns were prepared and the methanol fraction and 3 x 100 ml. fractions of 5N acetic acid in methanol were collected separately from each column. These fractions were evaporated and the Kober reaction applied in each case. The results corresponded with an apparent recovery of 2% of oestrone in the methanol fraction, an average of 40% in the first 100 ml. fraction of 5N acetic acid in methanol and of about 6% in the second 100 ml. fraction. The total apparent recovery was again of the order of 50%. These results suggested that most of the recovered oestrogen was being eluted from the column in accordance with the experimental procedure. However, in the case where oestrone-16-C^{14} was applied to similarly prepared columns, between 95-98% of the radioactivity was

recovered in the 5N acetic acid in methanol fraction. At this stage it was difficult to see why the apparent recovery of the oestrogen from the columns was so low.

It was decided, therefore, to examine more fully the analytical procedure with regard to purity of solvents and resin. Firstly, a batch of resin (200 g.) was subjected to a preliminary recycling by successive elution with 2N sodium hydroxide, distilled water, 2N hydrochloric acid and finally distilled water. Using columns prepared from this resin, the apparent recovery of oestrogens was still of the order of 50%. Secondly, a batch of resin (200 g.) was subjected to the full experimental procedure and regenerated by elution successively with 2N sodium hydroxide, distilled water, 2N hydrochloric acid and finally distilled water. Using columns prepared from this resin, the apparent oestrogen recovery was lowered to about 30% but there was a considerable reduction in extraneous absorption as evidenced by the lower extinction correction.

These results indicated that the analytical procedure was sound in so far as most of the radioactivity applied as oestrone-16-C^{14} could be recovered in the oestrogen-containing fraction. However, when applied to the quantitative determination of unlabelled oestrogens it appeared, from the low recoveries realised, that the development of the Kober colour was subject to inhibition by material present in the oestrogen-

containing fraction. In order to examine further this possibility, six columns were prepared to which no oestrogen was added and the 5N acetic acid in methanol fractions collected. These fractions were then evaporated, 10 micrograms oestrogen being added to each of three before evaporation. To each of the remaining three fractions, 10 micrograms of oestrone was added immediately before development of the Kober colour. The recoveries of added oestrogen lay within the range 26-37%. It can be concluded, therefore, from these results that the development of the Kober colour was being inhibited by material present in this fraction.

In order to obtain information as to which of the materials used contained inhibiting materials, a series of experiments was conducted, the results of which are summarised in Table XIII. The results showed that the inhibition of the development of the Kober colour was constant in (a), (b) and (d) but that inhibition decreased in (c) as the amount of methanol present before development of the Kober colour was reduced. Furthermore, in (d) the absorption maximum of the colour complex was shifted from 540 to 544 mμ.

Finally, it might be pointed out that, whereas the Kober colour complex usually exhibits a greenish-yellow fluorescence when developed by the Ittrich (1958) method, in all cases in this study the fluorescence was lacking to a greater or lesser extent. This indicated that the interfering material present inhibited the appearance of the

TABLE XIII

TABULAR SUMMARY OF EXPERIMENTS ON THE INHIBITING EFFECT OF RESIDUES FROM SOLVENTS ON THE ITTRICH-KOBER REACTION

Experiment performed	Oestrogen added	% recovery of added oestrogen as determined by the Ittrich modification of the Kober reaction
Evaporated to dryness 6 x 200 ml. 5N acetic acid in methanol	#1, 2 and 3, 10 μg. oestrone added before evaporation	37-38%
	#4, 5 and 6, 10 μg. oestrone added after evaporation	35-38%
Evaporated to dryness 200 ml., 100 ml., 50 ml. and 25 ml. 5N acetic acid in methanol respectively	10 μg. oestrone added to each before evaporation	43-45%
Evaporated to dryness that amount of methanol present in 200 ml., 100 ml., 50 ml. and 25 ml. 5N acetic acid in methanol respectively	10 μg. oestrone added to each before evaporation	33-76% (recovery improved stepwise with reduction in amount of methanol)
Evaporated to dryness that amount of acetic acid present in 200 ml., 100 ml., 50 ml. and 25 ml. 5N acetic acid in methanol respectively	10 μg. oestrone added to each after evaporation	25-36%

fluorescence as well as inhibiting the development of the Kober colour.

Even though the nature of the analytical difficulty appeared to have been determined, it was found that, even if a very high grade of acetic acid was used, the inhibition of the Kober colour complex could not be reduced to any great extent.

It was concluded that the technical difficulties presented by the presence of inhibitors of the Kober colour reaction in the solvents rendered this method unsuitable for the final purification of oestrogen extracts for colorimetric determination, whatever may be its merits for the purification of extracts intended for radioactive assay. Accordingly, attempts to use these resin columns were abandoned.

CHAPTER IV

AN ASSESSMENT OF THE RELIABILITY OF THE METHOD OF BROWN (1955) FOR THE QUANTITATIVE DETERMINATION OF OESTROGENS IN THE URINE OF THE DOMESTIC FOWL

A. INTRODUCTION.

Attempts to use ion-exchange chromatography as a purification step before the colorimetric measurement of avian urinary oestrogens having proved unsuccessful, it was decided to investigate the applicability of the Brown (1955) method.

This method, designed for determination of oestrogens in human urine, is the only method which has been applied successfully, either as such or with modifications, to the determination of urinary oestrogens in other species (Velle, 1958h; Lunaas, 1962; Raeside, 1961, 1963). Satisfactory results have been reported by these workers although they do not appear to have undertaken any extensive studies on the reliability of the method.

The study described below was undertaken in order to examine the reliability of the method as outlined by Borth (1952, 1957) in terms of its accuracy, precision, sensitivity and specificity.

B. METHOD.

The procedure for extraction, purification and determination of urinary oestrogens from avian urine has been outlined previously in Figure 9 and was used as such unless otherwise stated.

The Ittrich (1958) modification of the Kober reaction was carried out as described previously (Chapter II).

C. RESULTS.

1. ACCURACY.

The accuracy of the method was tested by adding 5 micrograms and 10 micrograms each of the reference standard preparations to 500 ml. pooled non-laying urine samples after hydrolysis. Two similar portions of the pooled urine were also analysed to correct the recovery figures for endogenous oestrogen content. The results of these investigations are presented in Table XIV.

2. PRECISION.

Results obtained from duplicate analyses of 48-hour non-laying urine samples were used to estimate the degree of precision for the measurement of the oestrone and oestradiol-17β fractions. According to Snedecor (1955) the precision of a method can be expressed as the standard deviation (s) as calculated from the difference between the two results of duplicate determinations in a large series of assays according to the formula:-

TABLE XIV

RECOVERY OF OESTROGENS ADDED TO POOLED AVIAN NON-LAYING URINE AFTER HYDROLYSIS

Oestrogen added	Amount added (μg)	Number of Experiments	Recovery % Mean ± S.E	Range
Oestrone	10	5	99.6 ± 0.8	97-101
	5	5	92.6 ± 1.6	89-98
Oestradiol-17β	10	5	92.5 ± 1.4	89-97
	5	5	94.0 ± 1.7	89-99
Oestriol	10	5	94.0 ± 0.8	91-96
	5	5	90.9 ± 1.3	86-94

$$s = \sqrt{\frac{\sum d^2}{2N}}$$

where d is the difference between the results of duplicate analyses and N is the number of duplicate determinations.

The values for s for oestrone and oestradiol-17β, derived from the results of duplicate analyses were 0.04 (N = 22) and 0.07 (N = 21) respectively, for oestrogen concentrations of less than 10 micrograms per sample.

The precision of the oestriol fractions was not checked due to the fact that in most of the non-laying urine samples analysed, the oestriol value for a 48-hour sample was invariably of the order of 0.5 micrograms.

3. SENSITIVITY.

The sensitivity of a method can be checked in two ways; either by recovery experiments with small amounts of standard substances, or by duplicate determinations at low concentration levels. If the estimate of precision at low concentration levels is s, then the sensitivity of a method can be calculated according to the formula:-

$$\text{Sensitivity} = \frac{t.s}{N}$$

where t is the tabulated t-value for N-1 degrees of freedom and N the number of duplicate determinations.

Furthermore, the error of a determination can be calculated from s as was done by Brown *et al.* (1957a) from the formula:-

$$\% \text{ error} = \pm \frac{100.t.s}{M \cdot N}$$

where M = mean of N single determinations. From these formulae and using the value of s calculated above, the smallest amounts of oestrone and oestradiol-17β which could be measured with a percentage error of not more than ± 25% (at P = 0.01) were 0.48 and 0.84 micrograms respectively in single estimations and 0.34 and 0.58 micrograms respectively in duplicate estimations. Similarly, the smallest amounts of oestrone and oestradiol-17β which could be distinguished from zero were 0.12 and 0.21 micrograms in single estimations and 0.08 and 0.15 micrograms in duplicate estimations.

4. SPECIFICITY.

Laying urine, corresponding to six twenty-four hour periods, was taken through the Brown (1955) procedure, as previously described (Figure 9). The oestrone methyl ether, oestradiol-17β methyl ether and oestriol methyl ether fractions from each twenty-four hour period were combined and the three fractions submitted to various tests.

a. Kober reaction spectra of the three oestrogen fractions.

The Ittrich (1958) modification of the Kober reaction was carried out on a portion of each of the three fractions. Figures 10, 11 and 12 show the wavelength/ absorption curves for (A) the oestrogen fraction, (B) the amount of pure oestrogen calculated to be present in this fraction by the correction formula of Allen (1950) and (C)

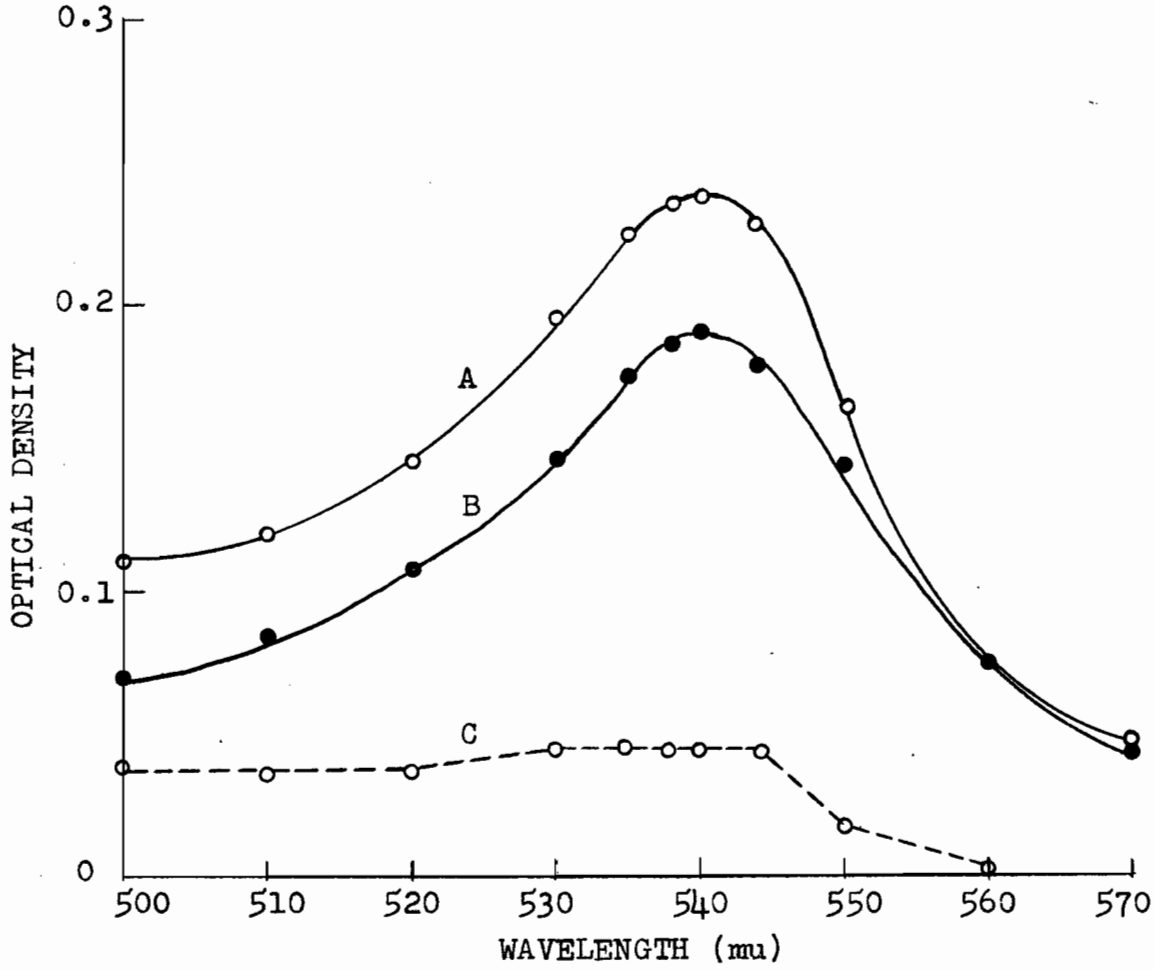


FIGURE 10. Wavelength/absorption curve for (A) the oestrone methyl ether fraction from urine; (B) the amount of pure oestrone methyl ether calculated to be present in this fraction by the correction formula of Allen (1950); and (C) the non-oestrogenic chromogenic material of the oestrone methyl ether fraction (A-B).

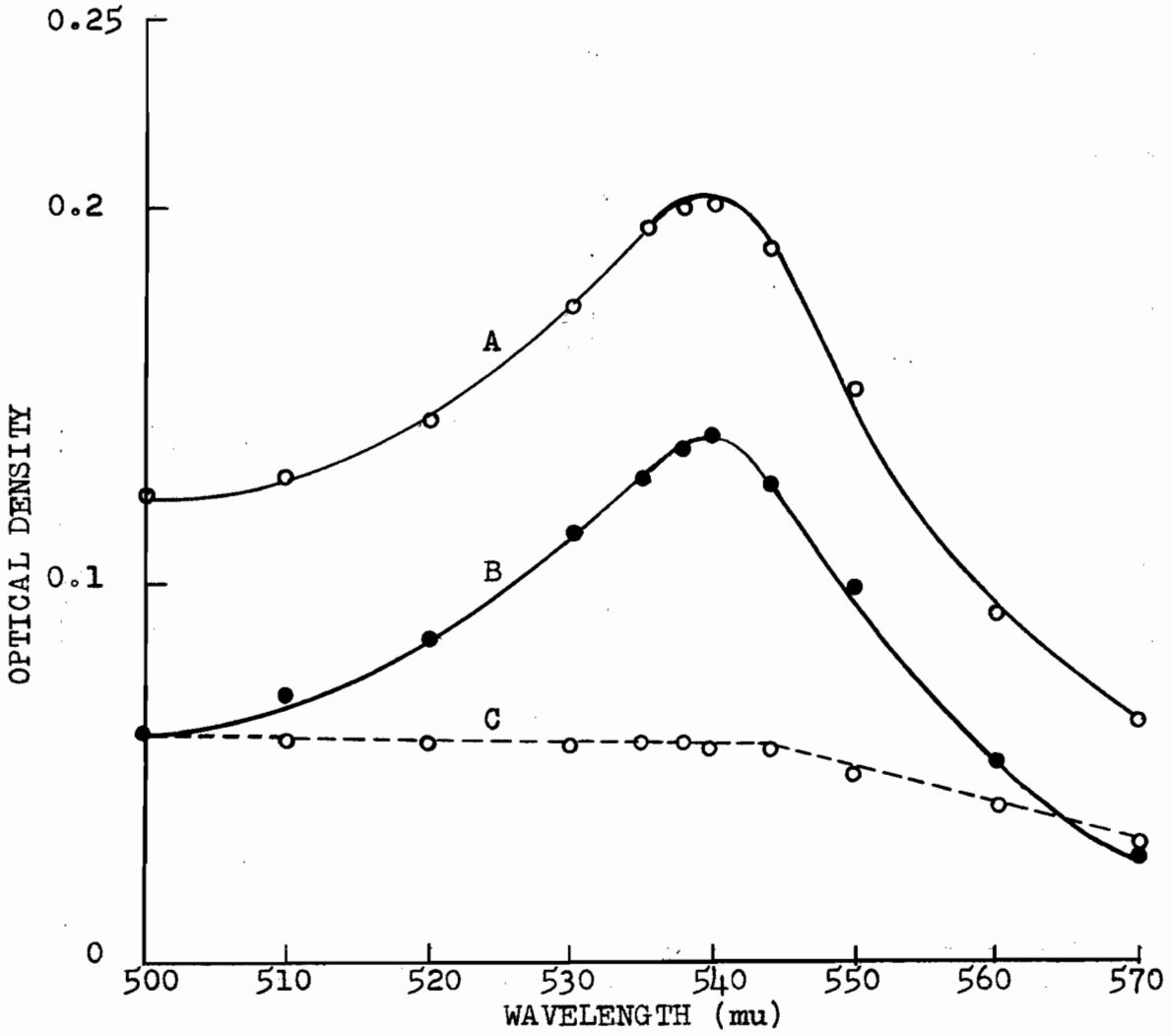


FIGURE 11. Wavelength/absorption curve for (A) the oestradiol-17β methyl ether fraction from urine; (B) the amount of pure oestradiol-17β methyl ether calculated to be present in this fraction by the correction formula of Allen (1950); and (C) the non-estrogenic chromogenic material of the oestradiol-17β methyl ether fraction (A-B).

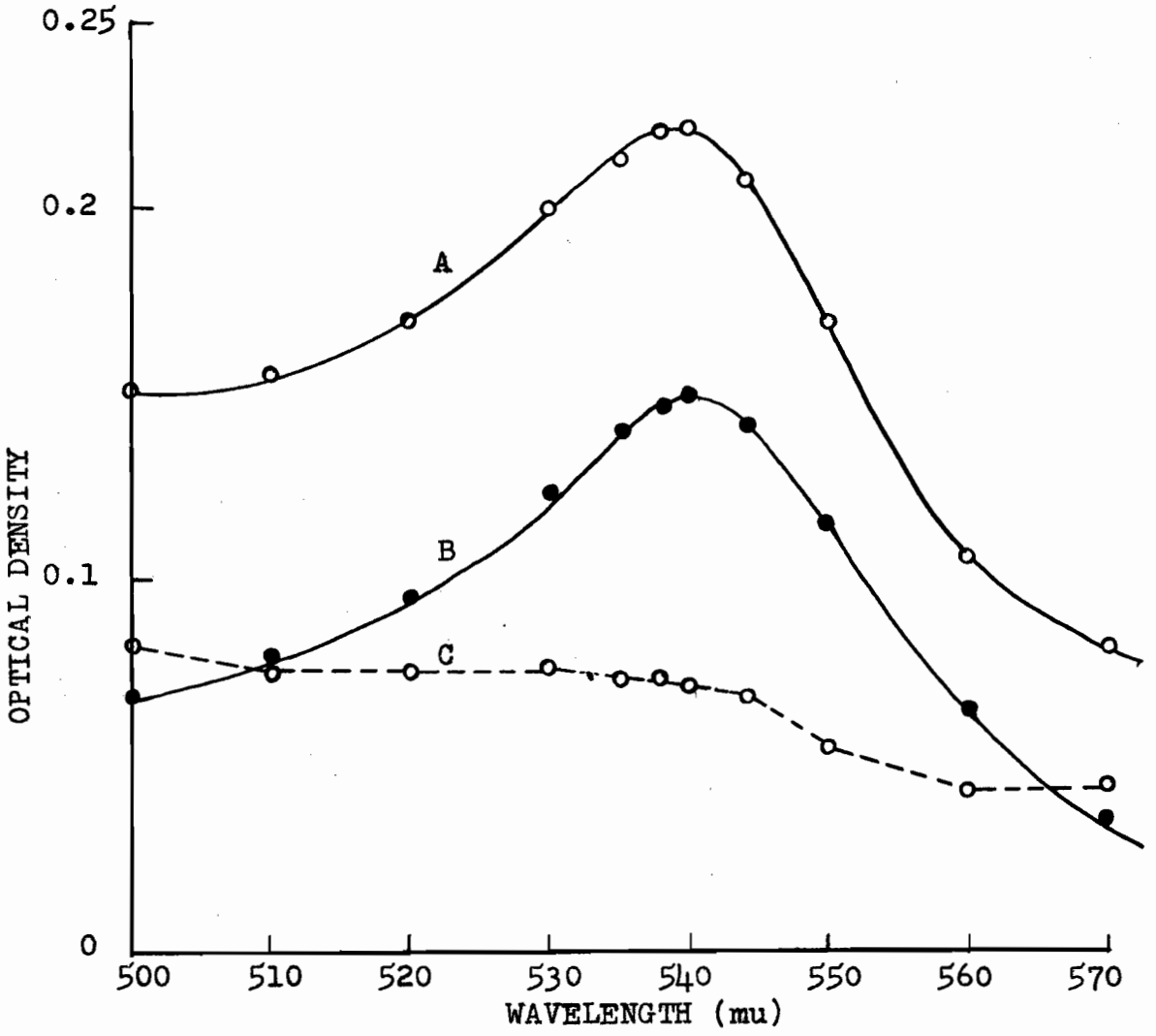


FIGURE 12. Wavelength/absorption curve for (A) the oestriol methyl ether fraction from urine; (B) the amount of pure oestriol methyl ether calculated to be present in this fraction by the correction formula of Allen (1950); and (C) the non-oestrogenic chromogenic material of the oestriol methyl ether fraction (A-B).

the non-oestrogenic chromogenic material of the oestrogen fraction (obtained by subtracting B from A). In no case was there an absorption peak between 500 and 580 mμ. for the non-oestrogenic chromogenic material. Similar results had been obtained previously for several 24-hour urine samples other than those discussed here.

b. Thin-layer chromatography of the three oestrogen fractions.

Thin-layer chromatography of the remainder of the three fractions was carried out on Silica Gel G as described by Hertelendy and Common (1963). Each fraction was chromatographed in a series of spots on a single chromatoplate along with spots of the appropriate reference oestrogen methyl ethers on either side. The positions of the spots following chromatography were revealed by staining lateral pilot strips on the chromatoplate with 2% sulphuric acid in 50% ethanol.

Oestrone methyl ether fraction. Thin-layer chromatography in the system benzene-methanol (95:5 v/v) revealed the presence of three spots following staining of pilot strips (Figure 13). Spot 3 corresponded in mobility to oestrone methyl ether. This spot was eluted with ethanol from the unstained portion of the chromatoplate and was then re-chromatographed in the system cyclohexane-methanol (97.5: 2.5 v/v) followed by drying and redevelopment in the same system. Spots 1 and 2 were eluted together with ethanol and the material corresponding to these spots was freed from ethanol by evaporation under nitrogen and the residue

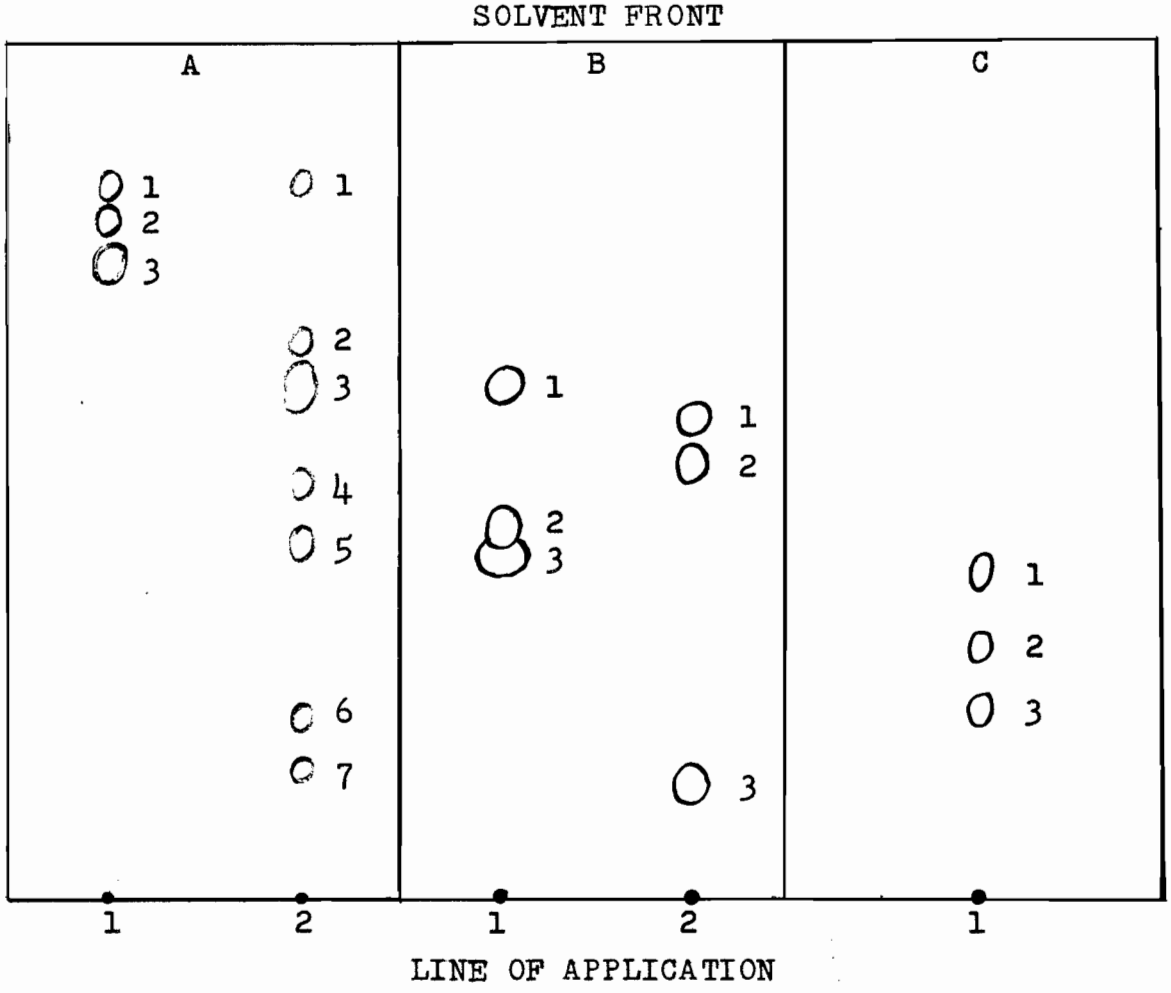


FIGURE 13. Thin-layer chromatography of the oestrogen methyl ether fractions following purification by the Brown (1955) procedure.

A. 1) Oestrone methyl ether fraction (system benzene-methanol, 95:5 v/v).

2) Material corresponding in mobility to oestrone methyl ether (system cyclohexane-methanol, 97.5:2.5 v/v).

B. 1) Oestradiol-17β methyl ether fraction (system benzene-methanol, 95:5 v/v).

2) 16-Epioestriol methyl ethers (system benzene-methanol, 95:5 v/v).

C. 1) Oestriol methyl ether fraction (system chloroform-ethanol, 85:15 v/v).

subjected to the Kober reaction. A negative Kober reaction was obtained. Rechromatography of the material having the mobility corresponding to spot 3 revealed six spots on staining of pilot strips (Figure 13). The third of these spots, which stained a distinct yellow colour, had the same colour and mobility as had the reference oestrone methyl ether. The remainder of the spots ranged from purple to violet in colour. The unstained portion of silica gel containing material having mobilities corresponding to each of the stained spots was scraped from the plate and eluted with ethanol. Following evaporation of the ethanol, each of the residues was subjected to the Kober reaction. The material with the mobility of oestrone methyl ether gave a positive Kober reaction, whereas the other materials did not.

Oestradiol-17β methyl ether fraction. Thin-layer chromatography in the system benzene-methanol (95:5 v/v) revealed the presence of three spots following staining of pilot strips (Figure 13). The most polar spot (spot 3), which stained yellow, had the same colour and mobility as that of reference oestradiol-17β methyl ether. However, this spot was contaminated with a slightly less polar spot which stained purple (spot 2). The least polar material (spot 1) stained a distinct red. The material corresponding in mobility to spot 1 and that corresponding to spots 2 and 3 were eluted separately from the unstained portion of the chromatoplate with ethanol, the ethanol evaporated and each

residue subjected to the Kober reaction. The material corresponding to spots 2 and 3 gave a positive Kober reaction. A similar positive reaction was obtained for the material corresponding to spot 1, although in this case the absorption maximum of the wavelength/absorption curve was at 538 mμ instead of at 540 mμ (Figure 14). Furthermore, the intensity of the Kober colour produced by the material corresponding to spot 1 was approximately twice that obtained with the material corresponding to spots 2 and 3.

It was suspected at this stage that the oestradiol-17β methyl ether fraction might be contaminated with the di-methyl ethers of 16-epioestriol which the author has found to be eluted from alumina columns in the oestradiol-17β methyl ether fraction. This is a possibility if one considers that only about 90% of any 16-epioestriol present will be removed from the benzene-hexane phase at the stage of the benzene-hexane-water partition (see Figure 9). Accordingly, the oestradiol-17β methyl ether fraction from two twenty-four hour urine samples was pooled and chromatographed by thin-layer chromatography in the system benzene-methanol (95:5 v/v) along with spots containing a mixture of the mono- and di-methyl ethers of 16-epioestriol. Following staining of pilot strips on the developed chromatoplate, it was observed that the 16-epioestriol dimethyl ethers were slightly more polar than the material corresponding to spot 1 (Figure 13). No further attempts

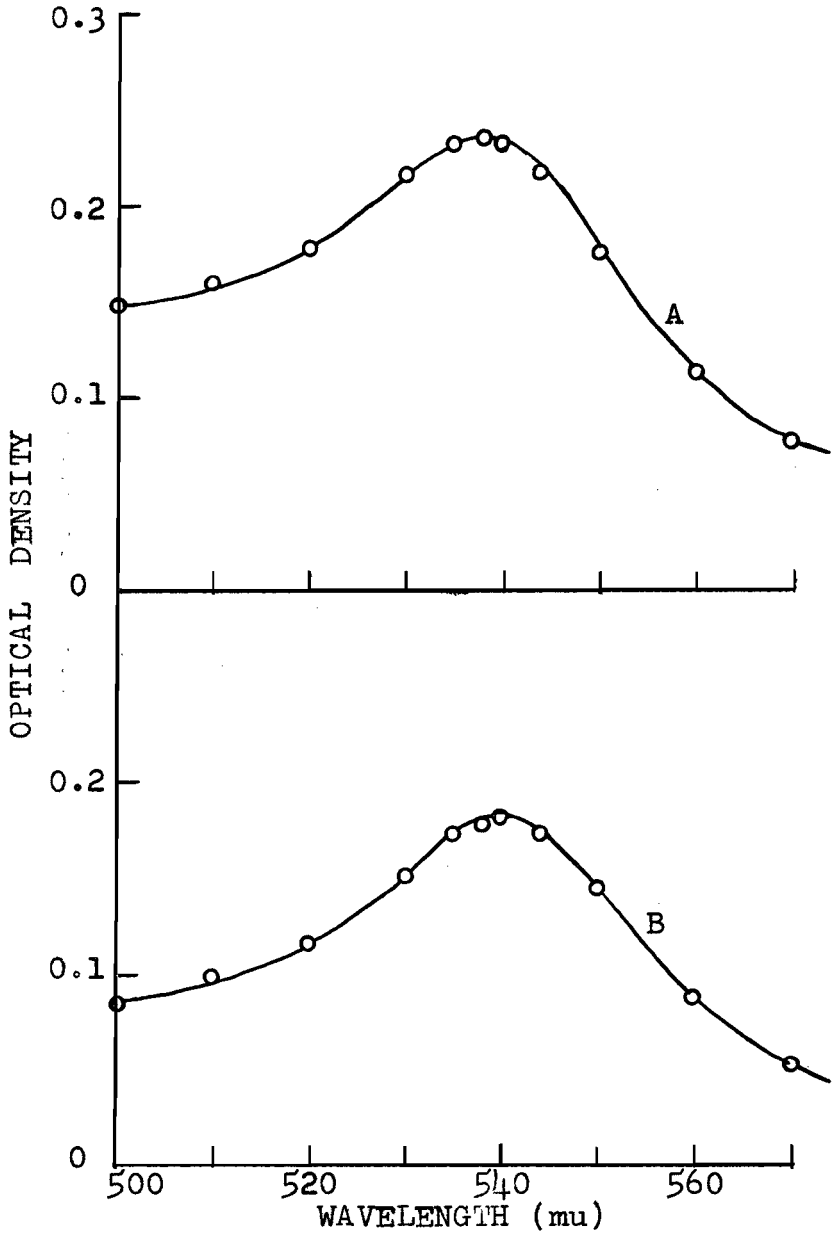


FIGURE 14. Wavelength/absorption curves for the interfering Kober chromogens present in (A) the oestradiol-17β methyl ether fraction, and (B) the oestriol methyl ether fraction.

have been made to identify this Kober positive contaminant.

Oestriol methyl ether fraction. Thin-layer chromatography in the system chloroform-ethanol (85:15 v/v) revealed the presence of three spots on staining of pilot strips (Figure 13). Spot 2 had a mobility corresponding to that of reference oestriol methyl ether although it appeared to be contaminated with some other material. The materials from unstained portions of the chromatoplate having mobilities corresponding to each of the three spots were eluted separately with ethanol, the ethanol was evaporated, and the residues corresponding to spots 1 and 3 were subjected to the Kober reaction. The material corresponding to spot 3 gave a positive Kober reaction (Figure 14) whereas that corresponding to spot 1 was Kober-negative. The material corresponding to spot 2 was rechromatographed by thin-layer chromatography in the system benzene-methanol (85:15 v/v), as a single spot, along with reference oestriol methyl ether. Staining of the chromatoplate revealed the presence of a spot having the same mobility as the reference oestriol methyl ether. However, whereas the reference material stained a violet-red colour, only a tinge of the same colour was evident in the spot corresponding to oestriol methyl ether from the urine extract, which was stained predominantly blue. There appeared, therefore, to be very little oestriol in the extract, which corresponded to three to four days of laying urine.

D. DISCUSSION.

The method of Brown (1955) developed for the determination of oestrone, oestradiol-17β and oestriol in human urine, appears to be applicable, with certain limitations, to the determination of avian urinary oestrone, oestradiol-17β and oestriol. The most serious limitations from a practical point of view relate to the accuracy of the values obtained for oestriol and oestradiol-17β. Thus, while thin-layer chromatography has provided confirmation of the presence of oestradiol-17β methyl ether in the oestradiol-17β methyl ether fraction, and has provided some indication of the presence of small amounts of oestriol methyl ether in the oestriol methyl ether fraction, nevertheless each of the methyl ether fractions contained a contaminant which also gave a positive Kober reaction. Moreover, the contaminants made a major contribution to the Kober colours obtained in each fraction.

It is of considerable interest that the contaminant of the oestriol methyl ether fraction, which was responsible for the greater part of the Kober colour given by this fraction, was more polar than oestriol methyl ether on thin-layer chromatograms. Whether this contaminant, and that observed in the oestradiol methyl ether fraction, are natural excretory products of oestrogen metabolism, or whether they are metabolites of dietary constituents is at present unknown. However, it has been reported that

Ittrich's modification of the Kober reaction is even more specific than the Kober colour itself. This is said to be especially true of the colour obtained with the methods using chloroform and tetrabromoethane (Ittrich, 1960). Thus, pending further experimental evidence, one cannot dismiss the possibility that the major part of the Kober colour given by the oestradiol methyl ether and oestriol methyl ether fractions may well represent oestrogen metabolites hitherto unrecognised in avian urine.

The thin-layer chromatographic studies on the oestrone methyl ether fraction showed that the only Kober positive material in this fraction was, in fact, oestrone methyl ether. Furthermore, the absorption due to non-oestrogenic chromogens in this fraction was less than that observed in either the oestriol methyl ether or oestradiol methyl ether fractions (Figures 10, 11 and 12). It may be concluded that Brown's (1955) method provides a reliable estimation of the oestrone content of avian urine.

Whilst the Brown (1955) method as applied to avian urine lacks specificity, save in respect to oestrone, the accuracy and reproducibility of the method (as determined by duplicate determinations) appear to be satisfactory. However, it was realised that this method is no longer reliable when the urinary oestrogen level falls below 0.5 micrograms per twenty-four hours. Since most of the urine samples obtained from both laying and non-laying birds contained oestrogen levels

above this limit, the method could be applied to the study of variations in urinary oestrogen excretion.

On the basis of these results it may be concluded that the method of Brown (1955) may be regarded as reliable for the determination of urinary oestrone in avian urine. Finally, this study exemplifies the difficulties which may be encountered in applying methods designed for human urine to urine from other species, and the necessity for *ad hoc* studies of accuracy and specificity in all such investigations.

CHAPTER V

A STUDY ON THE QUANTITATIVE EXCRETION OF OESTROGENS IN THE URINE OF THE DOMESTIC FOWL

A. INTRODUCTION.

Few attempts have been made to estimate urinary oestrogens for other than human subjects, and these have been confined almost entirely to urine from pregnant animals (Velle, 1958h). Recently, however, Lunaas (1962) using the Brown (1955) method and Raeside (1961, 1963) using a method based on the methods of Brown (1955) and of Bauld (1956) have determined the daily urinary oestrone excretion in sows during the oestrous cycle.

The effects of oestrogen administration to birds include many striking alterations in the biochemistry of blood, liver, bone, oviduct, etc. (Lorenz, 1954). The similarity of these changes to those which occur at puberty in the normal pullet clearly indicates the presence of endogenous oestrogens in the domestic fowl. Consequently, a quantitative study of the excretion of urinary oestrogens by the domestic fowl is of fundamental importance as a first step towards the investigation of endogenous oestrogen secretion in the fowl.

The aim of the present investigation has been to estimate the daily urinary oestrone levels in both non-laying and laying birds over a period of several months. It was hoped thereby to arrive at some correlation between the urinary oestrogen levels and the reproductive state of the experimental birds.

B. METHODS.

Twenty-four hour urine samples were collected from three White Leghorn hens which had been surgically modified so as to provide them with exteriorized ureters. Samples were collected from each bird throughout the summer months (May - September) although during May and June the urine collections were interrupted. Thereafter, between 18 to 20 daily urine samples were collected each month, usually in cycles of three to four days. The urine samples were kept in the deep-freeze until analyses could be undertaken, which was usually within six days after collection.

Separation and purification of oestrone, oestradiol-17β and oestriol from the urine samples were made using the method of Brown (1955). Quantitative determinations of the amounts of oestrogen in the purified fractions were made using the Ittrich (1958) modification of the Kober reaction.

C. RESULTS.

The levels of urinary oestrone excretion by Birds 2 and 3 over the period May - September are illustrated in Figure 15. Noteworthy are the following:-

a. The rise in the level of oestrone excretion over the the two weeks immediately before laying of the first egg.

b. The consistency with which the urinary oestrone level remained during the laying period at a higher level than during the non-laying period.

c. The sharp drop in the level of urinary oestrone excretion that took place immediately after the laying of the last egg of a prolonged laying period.

A further point of interest is the rhythmic rise and fall in the level of urinary oestrone excretion that appeared to take place during the non-laying period.

As explained in Chapter IV, the precise significance of the observed values for excretion of oestradiol-17β and of oestriol are doubtful. However, if the observed results for oestradiol-17β and for oestriol are set out as in Figure 16, the same general pattern is evident in the excretion of all three oestrogen fractions measured. Table XV shows mean values and standard deviations for the urinary oestrone fractions from the three experimental birds. In all cases the results shown are uncorrected for methodological losses incurred in the method of determination.

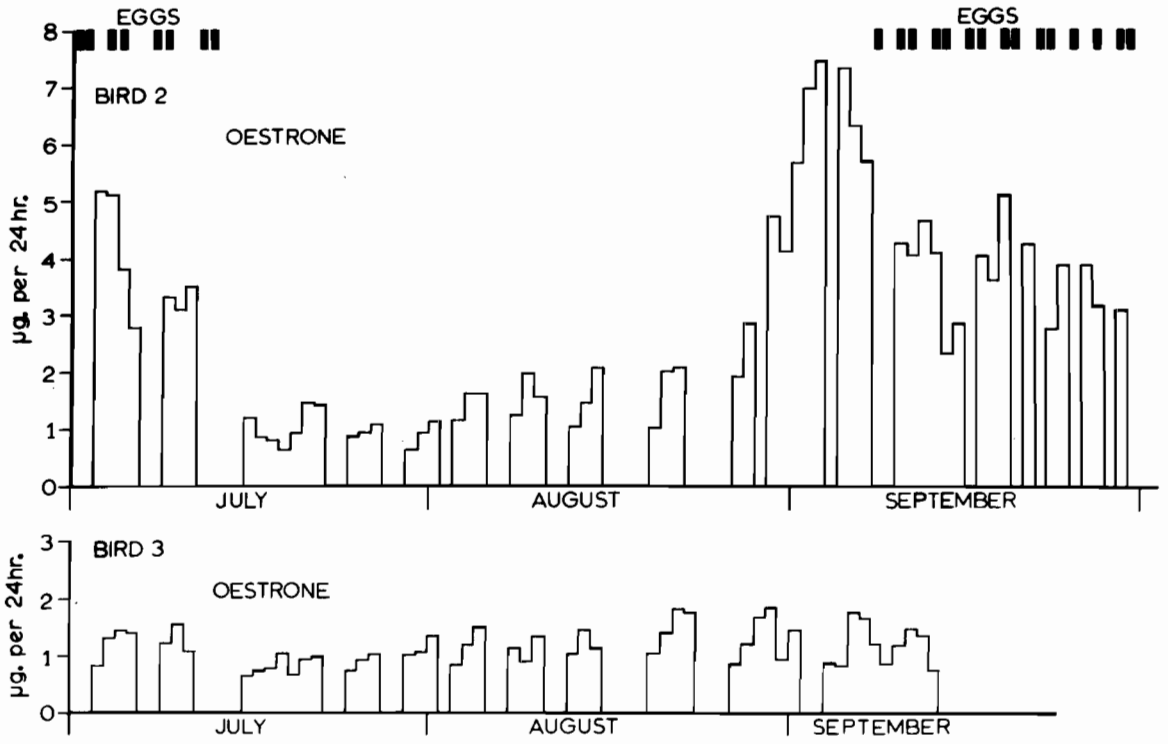


FIGURE 15. URINARY OESTRONE EXCRETION (PER 24-HOURS) BY TWO DOMESTIC HENS DURING THE PERIOD JULY - SEPTEMBER 1963 INCLUSIVE. (The values obtained during the months May and June have been omitted since only twelve 24-hour urine collections were made over this period).

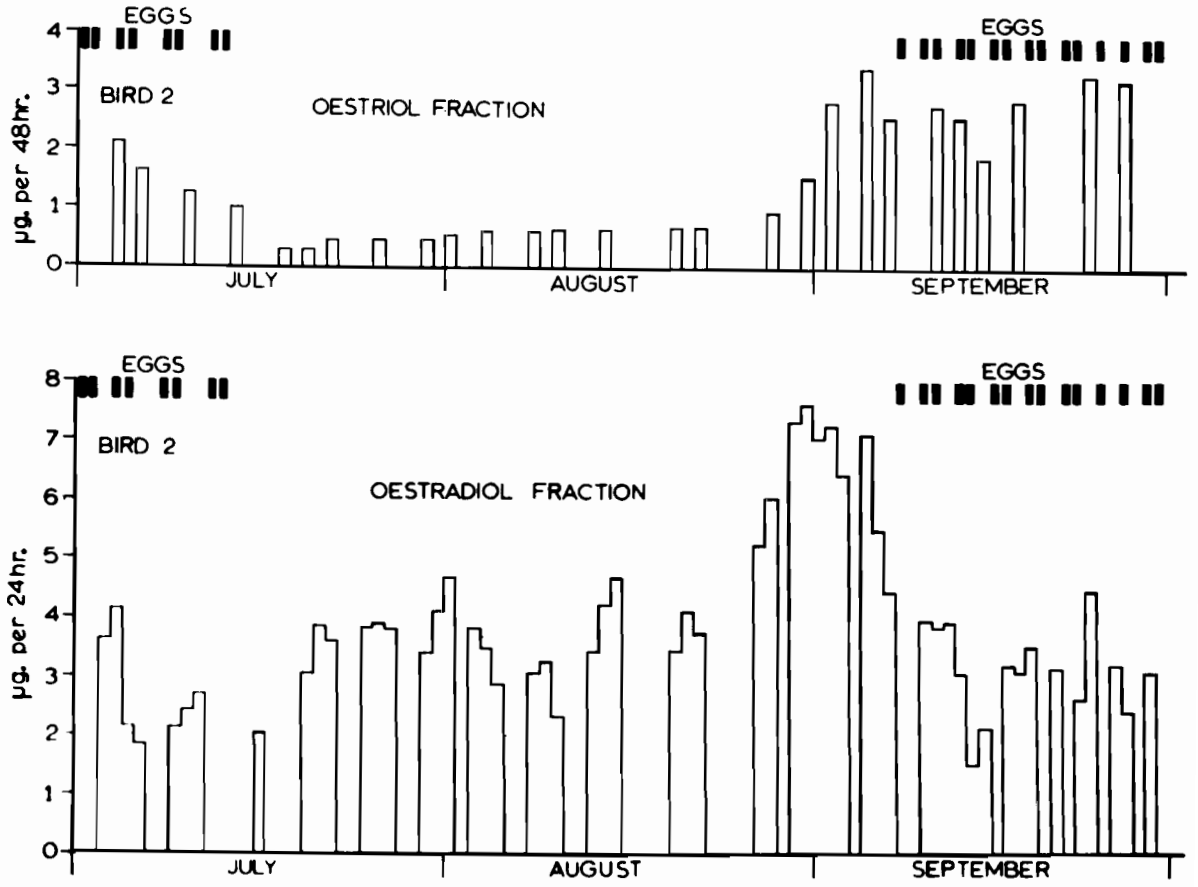


FIGURE 16. EXCRETION OF KOBER POSITIVE MATERIAL IN THE URINARY OESTRIOL FRACTION (PER 48-HOURS) AND THE URINARY OESTRADIOL-17β FRACTION (PER 24-HOURS) BY BIRD 2 DURING THE PERIOD JULY - SEPTEMBER 1963 INCLUSIVE.

TABLE XV

URINARY EXCRETION OF OESTRONE BY THE DOMESTIC FOWL

(Expressed as micrograms/24 hours;

range in parentheses).

Bird No.	Number of determinations	$\bar{x}$	$S.D._x$	$S.D._{\bar{x}}$
1 (non-laying)	29	0.71 (0.39-1.04)	0.23	0.04
2 (non-laying)	25	1.27 (0.63-2.07)	0.44	0.09
2 (laying)	27	4.06 (2.31-5.19)	0.77	0.15
3 (non-laying)	50	1.15 (0.5-1.84)	0.34	0.05

D. DISCUSSION.

The appraisal of the reliability of the fractionation technique employed indicates that it has been possible to achieve to a limited extent the main objective of this study, namely to obtain a fairly reliable picture of the quantitative excretion of oestrogen in normal avian urine. However, although a definite increase in all three urinary oestrogen fractions was observed in the two weeks prior to laying in Bird 2, it was only in the oestrone levels that a true increase was demonstrated with any degree of reliability, due to the fact that the increases in the oestradiol-17β and oestriol levels may have been due to other Kober positive materials which have been shown to contaminate these fractions. At this point, the identity of these Kober-positive contaminants is unknown, and it would be unwise to state that the rise in these levels was due to an increase in oestrogen excretion. However, there is no doubt that in the ten to twelve days preceding the start of a laying period, there was a marked increase in the total urinary oestrogen, as evidenced by an increase of Kober positive material in all three urinary fractions.

This definite increase in urinary oestrogen levels in the ten days prior to the onset of laying correlates closely with the pronounced increase in total serum calcium and phosphorus (Heller *et al.* 1934) and plasma total lipid, free fatty acid and phosphoprotein (Heald and Badman, 1963).

Furthermore, in the latter case it was shown that the quantities of these components decreased markedly when laying commenced. This fact has also been demonstrated in Figures 15 and 16 for the urinary oestrogen levels. It would appear, therefore, that as the female fowl approaches maturity, or the onset of a laying period, the urinary oestrogen levels follow very closely the growth and maturation of the ovarian follicles which begin growing at a rapid rate and reach maturity within nine to ten days (Warren and Conrad, 1939). The oestrogen elaborated by the growing follicles and medulla of the ovary then produces the well-known and pronounced changes in the oviduct and blood chemistry (see Lorenz, 1954; Sturkie, 1958; van Tienhoven, 1959, for a more extensive documentation of the various aspects of these changes).

CHAPTER VI

SUMMARY - PART II

1. Attempts to use ion-exchange chromatography as a means of purification and separation of oestrogens from interfering materials were unsuccessful. Although excellent recovery of radioactive oestrone from the column was realised, attempts to quantitate unlabelled oestrogens from the column by use of the Kober reaction were unsatisfactory because of the presence of material or materials which inhibited the development of the Kober colour.

2. An assessment of the Brown method for the estimation of oestrogens in avian urine showed the method to be satisfactory within certain limits. The reliability of the method was investigated and particulars concerning its accuracy, precision, sensitivity and specificity are presented. The specificity studies on the oestrone, oestradiol-17β and oestriol methyl ether fractions using thin-layer chromatography indicated that the latter two fractions contained a Kober positive contaminant, in each case, in addition to oestradiol-17β and oestriol respectively; both of these contaminants were found to contribute greatly to the final Kober colour. The implications of the above findings for estimations of urinary oestrogens by the method of Brown are discussed.

3. Quantitative estimation of urinary oestrone excretion was carried out in three birds using the method of Brown. The method yielded the following results: Bird 1 (non-laying), 0.71 micrograms per 24-hours (range 0.39-1.04); Bird 2 (non-laying), 1.27 micrograms per 24-hours (range 0.63-2.07); Bird 2, (laying), 4.06 micrograms per 24-hours (range 2.31-5.19); Bird 3 (non-laying), 1.15 micrograms per 24-hours (range 0.5-1.84). The pronounced and rapid increase in oestrogen excretion which was observed during the ten days prior to the laying of the first egg of a laying period is discussed in relation to the effects of oestrogen on the composition of blood and other tissues.

CLAIMS TO ORIGINAL RESEARCH.

1. The isolation of crystalline oestrone from the urine of the hen and its characterization by infrared spectroscopy. The author believes this to be the first reported isolation, in crystalline form, of a naturally-occurring steroid oestrogen from avian urine.

2. The isolation of crystalline equol from the urine of the hen and its characterization by infrared spectroscopy. The author believes this to be the first reported isolation of equol from avian material.

3. The demonstration that injected 16-oxooestradiol-17β-16-C^{14} is converted *in vivo* by the domestic fowl into radioactive 16-epioestriol, as evidenced by its isolation from avian urine and subsequent purification to constant specific activity.

4. The demonstration that 16-epioestriol-16-C^{14} is converted *in vivo* by the domestic fowl into radioactive 16-oxooestradiol-17β, as evidenced by the isolation of the latter from avian urine and its subsequent purification to constant specific activity. As far as the author is aware, this work constitutes the first study on the *in vivo* conversion of a ring D α-ketolic oestrogen and of its primary conversion product in a species other than the human.

5. The demonstration that 16-oxooestradiol-17β-16-C^{14}, when injected into the domestic fowl, appears to be preferentially reduced to radioactive 16-epioestriol. This result is believed to be the first demonstration of a qualitative difference between the metabolism of oestrogens in man and in the domestic fowl.

6. (a) Provision of quantitative chemical estimates of the daily excretion of urinary oestrogens by application of a slightly modified version of the method of Brown (1955).

   (b) The demonstration, by the foregoing means, that daily oestrone excretion by the hen rose from a value of ca. 0.6-2.0 micrograms per day before laying to a value of ca. 2.3-5.2 micrograms when laying began.

   (c) The demonstration that the increase of daily oestrone excretion from the pre-laying level took place over a period of about 10 days before the laying of the first egg.

To the best of the author's knowledge the foregoing observations 6(a), 6(b), 6(c) constitute the first quantitative chemical information on daily urinary oestrogen excretion by an avian species.

PART III

APPENDIX

A MODIFIED SURGICAL TECHNIQUE FOR THE EXTERIORIZATION OF THE OPENINGS OF THE FOWL'S URETERS.

A. INTRODUCTION.

Metabolism studies in avian species have been handicapped in the past because of the complication that the urine and faeces are voided in admixture. However, several techniques have been devised for separate collection of urine and faeces, and some of these are now coming into regular use for experimental purposes.

As early as 1886, Minkowski (1886) reported a method whereby urine could be collected for short intervals. The technique involved ligation of the rectum anterior to the cloaca of anaesthetised geese. Milroy (1904) reported a similar technique in which an artificial anus was created to prevent faecal contamination of the urine during longer periods of study.

More recently, techniques have been reported in which urine and faeces can be collected over a long period of time. Dixon (1958) and Richardson et al. (1960) have described techniques for exteriorization of the rectum in which a glass cannula was used to prevent the surrounding skin from encroaching on the artificial anus. A low fibre diet was recommended if the birds were to be used indefinitely. A somewhat similar technique has been developed by Fussel (1960); in this technique, the terminal end of

the large intestine as it joins the cloaca was ligated and exteriorized at a point half-way between the ischium and the abdominal mid-line and approximately 1½ inches anterior to the ventral lip of the cloaca on the right side.

Hester et al. (1940) developed a technique for exteriorization of the ureters of hens in which the dorsal portion of the cloaca was elevated on the pygostyle. Hart and Essex (1942) exteriorized the ureters dorsal to the vent through a fistulous tract, a procedure which proved to be superior to the creation of an artificial anus, particularly for long term studies.

The surgical technique described herein is similar to that described by Dixon and Wilkinson (1957), except that the operation is performed in a single stage and a different technique is used to close the wound after isolating the mucosa containing the ureteral openings from the mucosa of the cloaca.

B. METHODS.

1. ANAESTHESIA.

A general anaesthetic is used. In the early stages of the work Nembutal ('Pentobarbital', obtained from Stevenson, Turner and Boyce Ltd., Guelph, Ontario, containing 65 mg. pentobarbital sodium per ml.) was used to anaesthetize the birds. However, its duration is short; the bird begins to recover five to fifteen minutes after a full anaesthetic dose. Furthermore, the exact amount is critical

and a small overdose is rapidly fatal.

In the later stages of the work, phenobarbitone given intramuscularly following induction with Nembutal intravenously, has proved very satisfactory. For induction, Nembutal is injected slowly into the wing vein until the neck muscles relax and the eyes close. The comb should be pinched from time to time and more Nembutal given until the response to pinching is feeble. The amount of the Nembutal preparation required to reach this stage varies between 0.5-1.0 ml. depending on the weight of the bird. 60 mg. Phenobarbitone ('Luminal Sodium', obtained from Winthrop) is then injected into the breast muscle. Under these conditions the bird may be kept under moderate anaesthesia for at least one hour, usually longer. In cases where the anaesthesia is not of sufficient duration, ether can be used to maintain the anaesthesia with no special difficulties so long as vigilance is observed.

2. SURGICAL TECHNIQUE.

The operation involves the exteriorization of the ureters and their attachment to the skin just dorsal to the vent; this is followed by complete isolation of the ureters from the cloaca.

The operative procedure can be conveniently described in four steps as follows:

1. An initial, vertical, midline incision is made through the skin, perineal muscle, and the dorsal mucosa of the cloaca (Figure 17). The incised edges of the vent are retracted laterally and the incision is extended dorsally through the skin and perineal muscle. A small, circular piece of skin and fatty tissue is then dissected out at the dorsal end of the incision (Figure 18).

2. The urodeum is then retracted posteriorly and an incision is made anterolaterally on each side to a point just lateral to each ureteral opening, and the tissue scraped back using blunt dissection (Figure 19). Care must be taken when making this incision not to sever a ureter or to penetrate the peritoneum. Simple interrupted mattress sutures with No. 000 braided silk are used to unite the reflected mucosa of the urodeum to the skin just below the pygostyle (Figure 19).

3. Starting near the midline, a tension suture is placed in the mucosa on each side, on a line midway between the ureteral openings and the opening of the oviduct. Using these tension sutures for retraction, a transverse incision is made through the mucosa between the tension sutures and the opening of the oviduct (Figure 20). The mucosa is then reflected posteriorly approximately 1/8 inch by blunt dissection, again exercising care to avoid severing a ureter or penetrating the peritoneum. The mucosa containing the ureteral openings is now completely isolated from the mucosa of the cloaca.

4. To close the wound, the cut edges of the vent are freshened and cut back so that excess tissue is removed. Each side of the vent can then be sutured with simple interrupted mattress sutures to the dorsal mucosa of the cloaca between the isolated ureters and the anus (Figure 21). The wound in the dorsal lip of the vent is then closed with simple interrupted mattress sutures (Figure 22). Finally, simple interrupted sutures are used to unite the isolated mucosa surrounding the ureteral openings to the skin.

3. POST-OPERATIVE CARE.

Following surgery, the birds receive an intra-muscular injection of a long acting, broad range antibiotic. 'Mypen' ointment (a penicillin-streptomycin preparation, supplied by Ayerst, McKenna and Harrison, Montreal) is spread over the wound every twelve hours for the five days following surgery. The wound is washed with distilled water at least four times per day to remove solid urate material, which tends to collect in the area, and to prevent mechanical blocking of the ureteral openings. The sutures are removed after five to seven days and the birds allowed to move freely in the cages for at least two weeks. This permits complete healing of the wound, after which, provided the operation is successful, urine can be collected.

At this stage it is desirable to draw attention to the difficulties which are encountered following surgery. The greatest difficulty encountered by the present author has arisen from incomplete union of the mucosa of the cloaca

with the tissue of the dorsal lip of the vent. When this occurs, a fistula develops such that the urine from the ureteral openings, instead of running down on the outside of the vent, runs down on the inside and becomes contaminated with faeces excreted *via* the anus. In this case, the bird may either be discarded or re-operated, depending on the seriousness of the fistula. A second difficulty encountered, especially in the four days succeeding the operation, has been a tendency for the ureters to become blocked with solid urate material. This can be avoided to a great extent by repeated washing of the wound with distilled water to prevent accumulation of solid urates. However, after healing of the wound has taken place, cleaning of the area can be cut down to twice daily, in the morning and in the evening. If the operation is successful, the bird may be used indefinitely.

4. COLLECTION OF URINE.

After post-operative recovery and healing of the wound a urine collection apparatus, composed of a canvass harness, can be attached to the bird to hold the urine collection bag in place. The urine collection bag is made from 2-inch diameter latex tubing (rubber artificial vaginal liners, obtained from Central Sales Ltd., Brampton, Ontario). One end of this is sutured to the tissue between the ureters and the vent and held in place by string which is attached to the harness. The tubing is folded and the opposite end closed with string which is attached to the

harness. Collection of the urine is then made twice daily. Usually the collection bag will remain in place from four to six days, after which it is removed and a clean bag attached in its place. If necessary, faeces can be collected by putting dropping pans underneath the cages.

Finally, attention might be drawn to the physical characteristics of the urine samples. Urine collected from non-laying birds is pale yellow in colour with the solid urate material assuming a white colour. However, in laying birds the urine tends to be slightly darker whilst the urate material takes on a definite yellow tinge. This yellow colour associated with the urate material disappears again within twenty-four hours of the laying of the last egg of a laying period.

C. DISCUSSION.

The author's experience with the Dixon and Wilkinson (1957) technique for exteriorization of the ureters of the hen included a high incidence of fistulation following surgery. This was due principally to incomplete union of the mucosa of the cloaca with the dorsal tissue of the vent. However, a second factor which tended to hinder the complete healing of the wound was the excessive amount of tissue which was present in the area. The operation as modified by the author, eliminates much of this excess tissue and facilitates the healing of the mucosa. This has reduced the incidence of fistulation

by an estimated 50 per cent, although casualties following surgery are still of the order of 25 per cent.

It may be remarked that White Leghorn and Rhode Island Red hens have been equally amenable to surgery. However, surgically modified Rhode Island Red hens have proved to be much more useful for experimental purposes. Laying is usually reestablished within four weeks of operation in the case of Rhode Island Red hens whereas the re-establishment of laying in the case of Leghorn hens may take up to four months or occasionally be postponed to all intents and purposes indefinitely. This outcome is no doubt related to the more 'nervous' constitution of the Leghorn breed.

DIAGRAMMATIC REPRESENTATION AND ACTUAL PHOTOGRAPHS OF THE MODIFIED SURGICAL TECHNIQUE FOR EXTERIORIZATION OF THE URETERAL OPENINGS OF THE FOWL

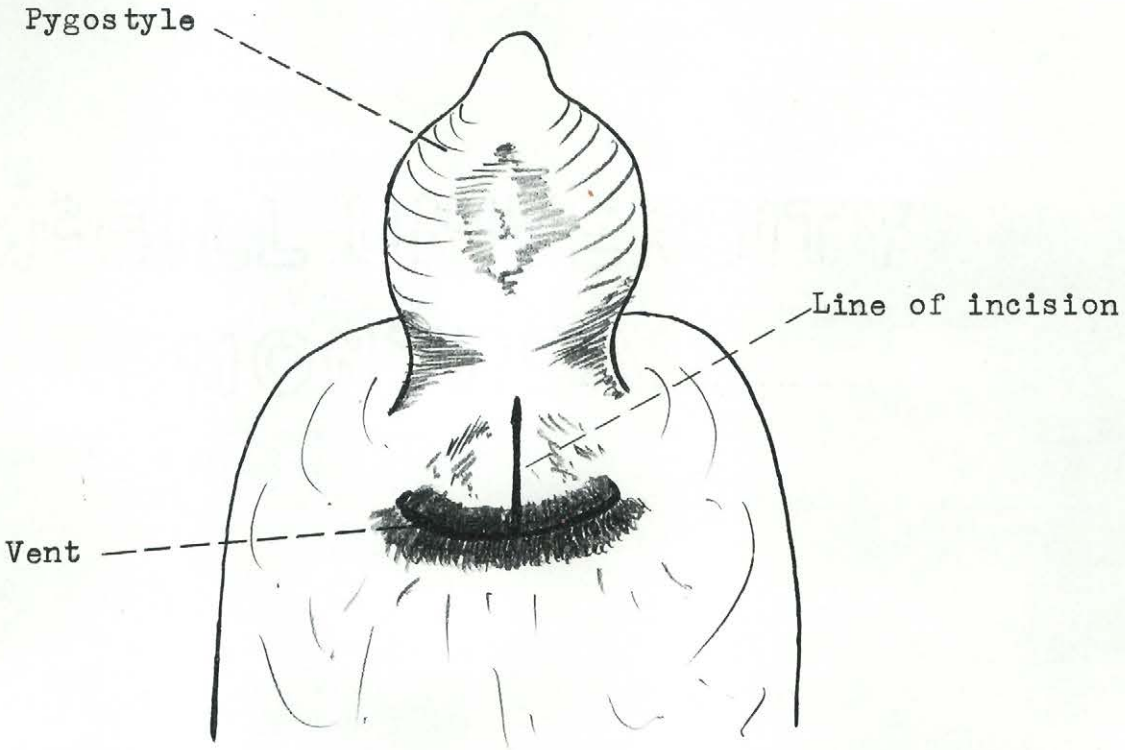


FIGURE 17: Step 1.

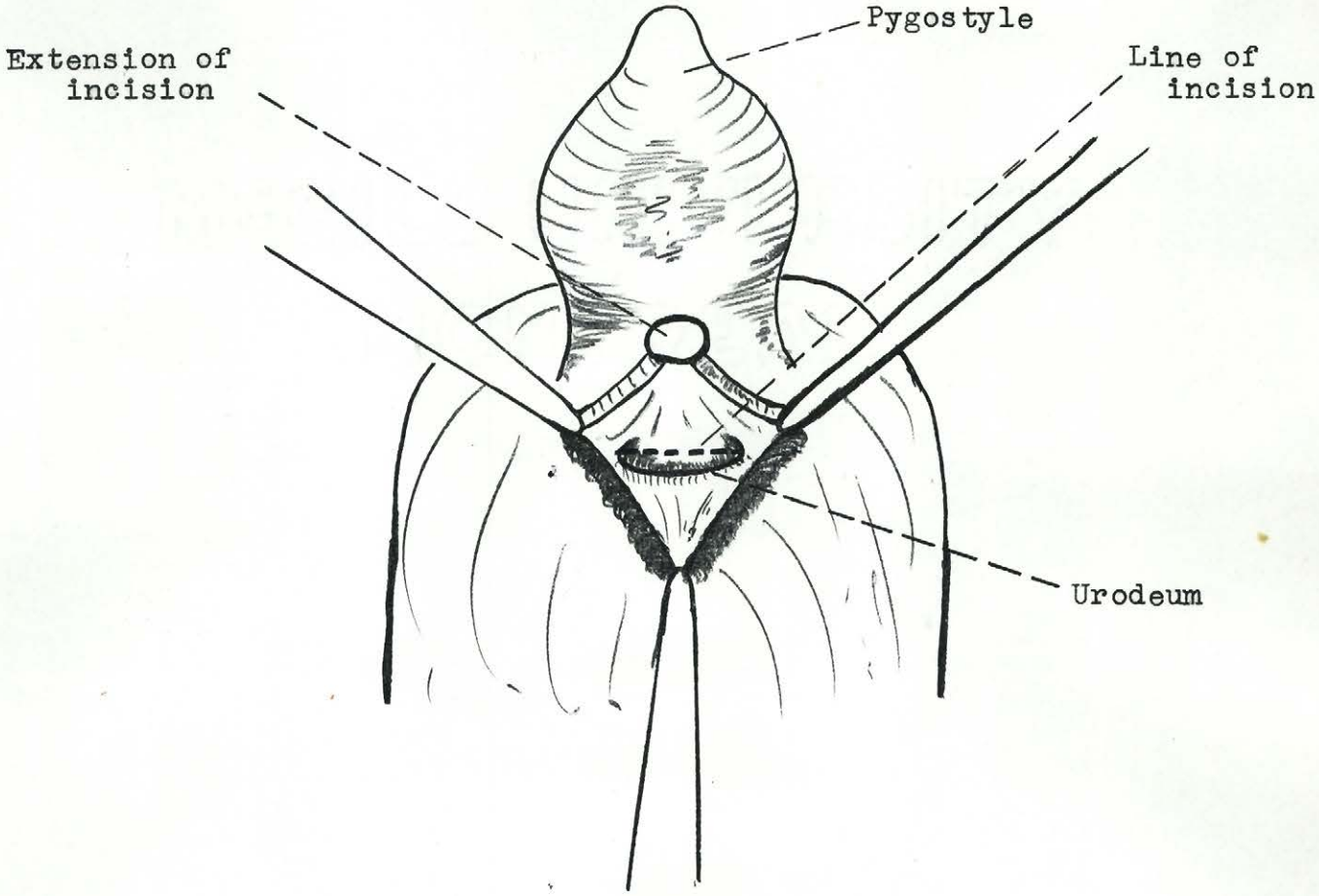


FIGURE 18: Step 1.

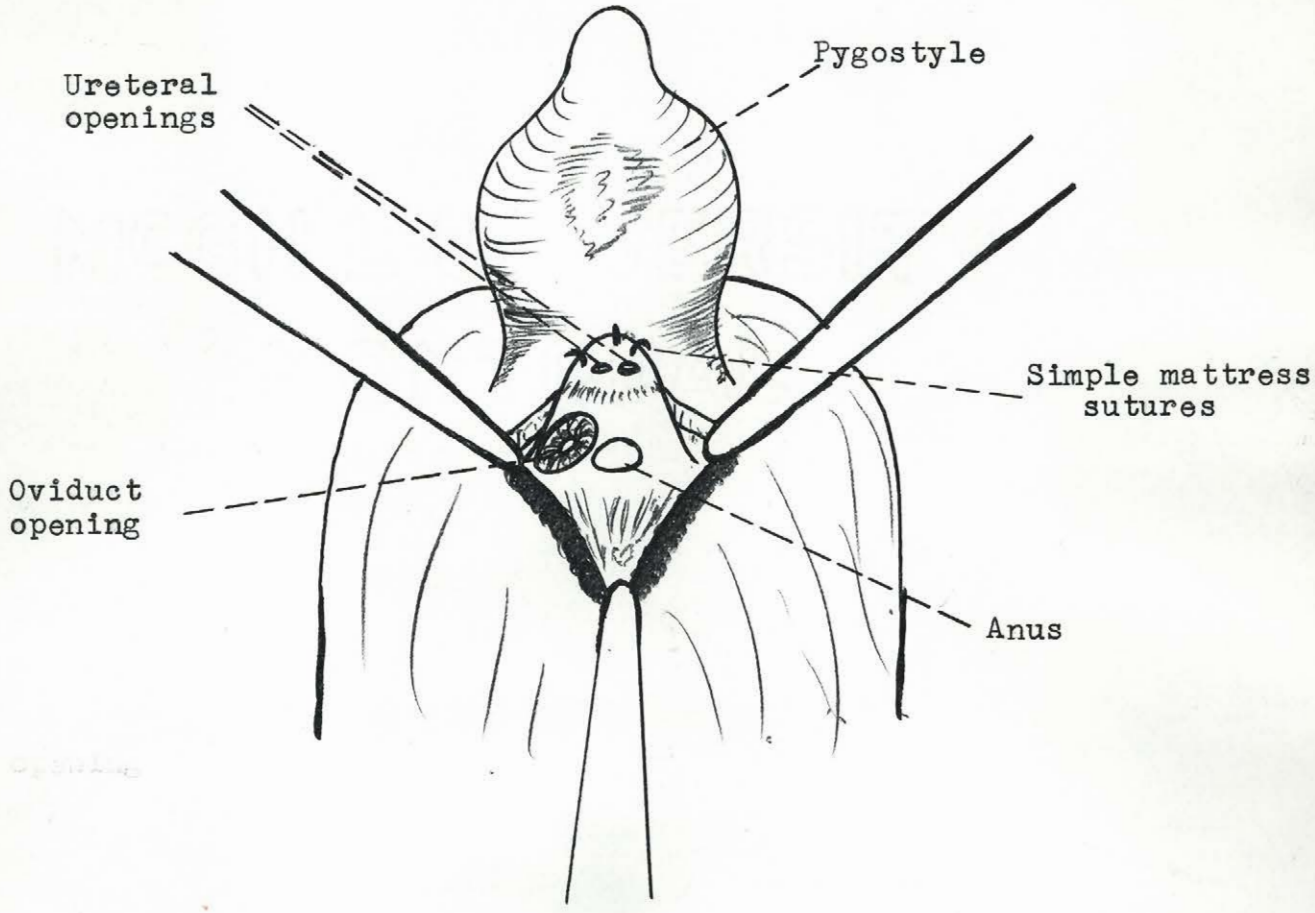
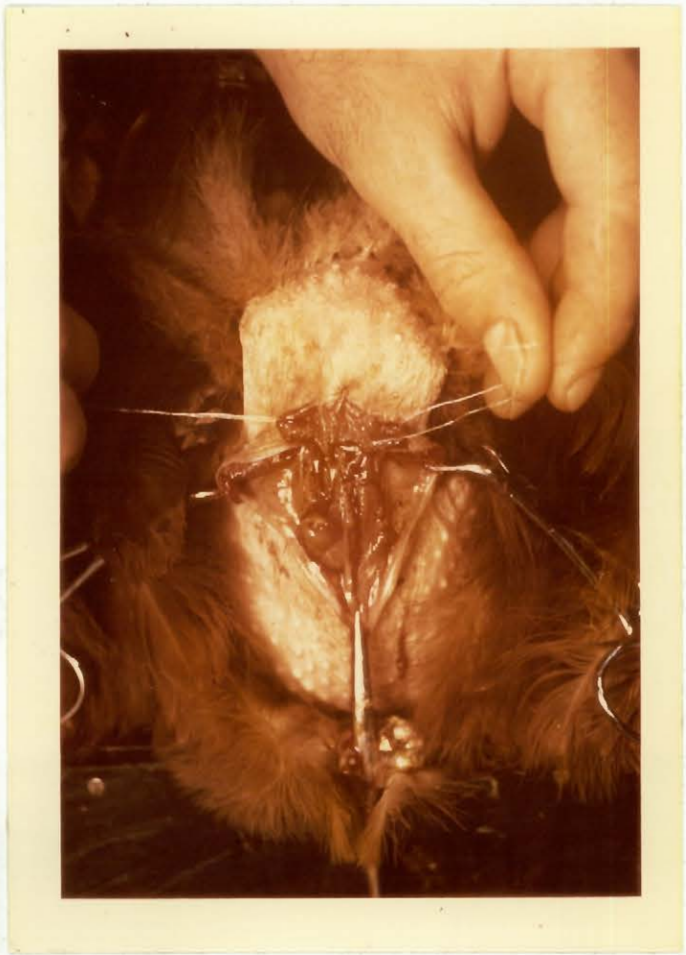


FIGURE 19: Step 2.



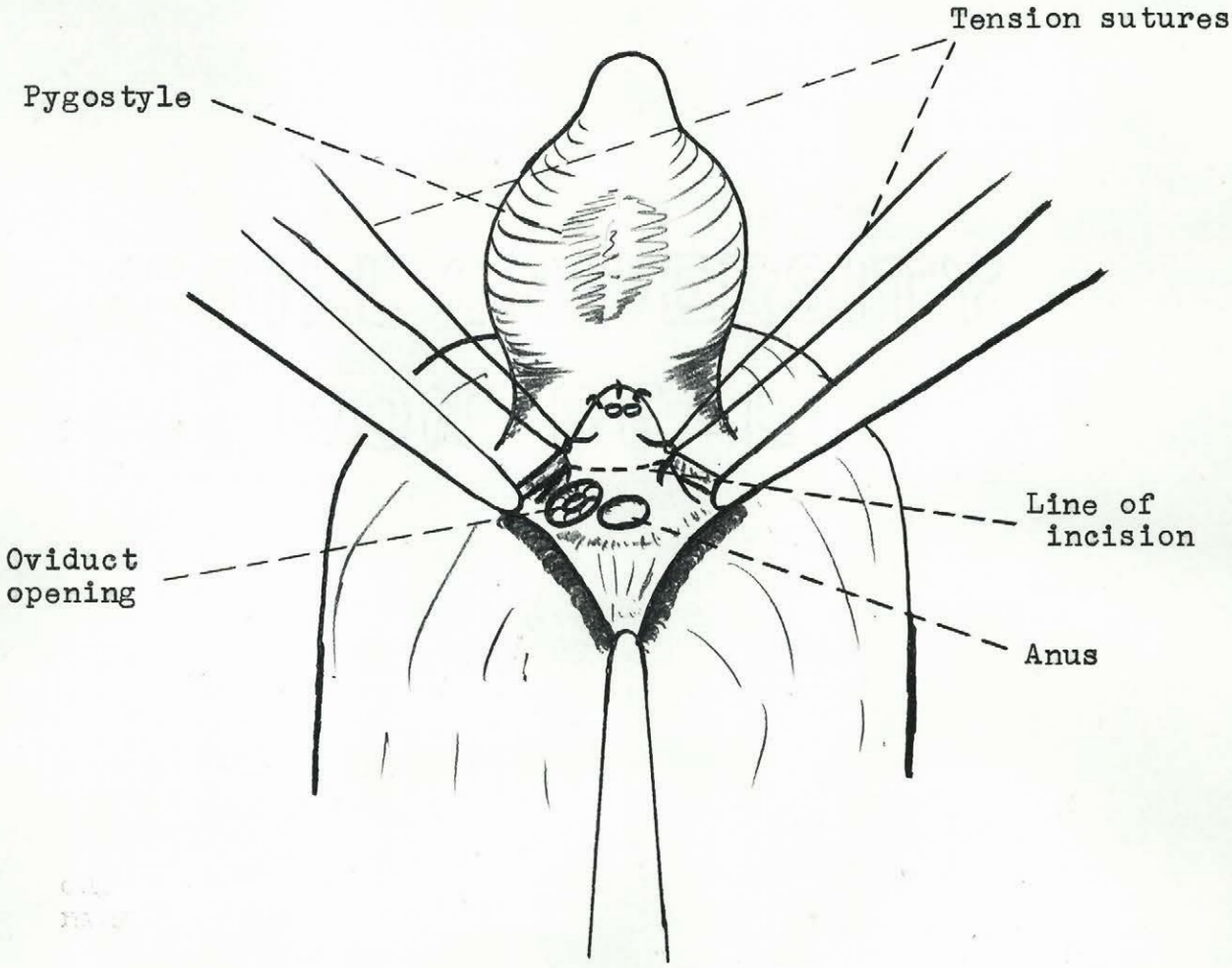
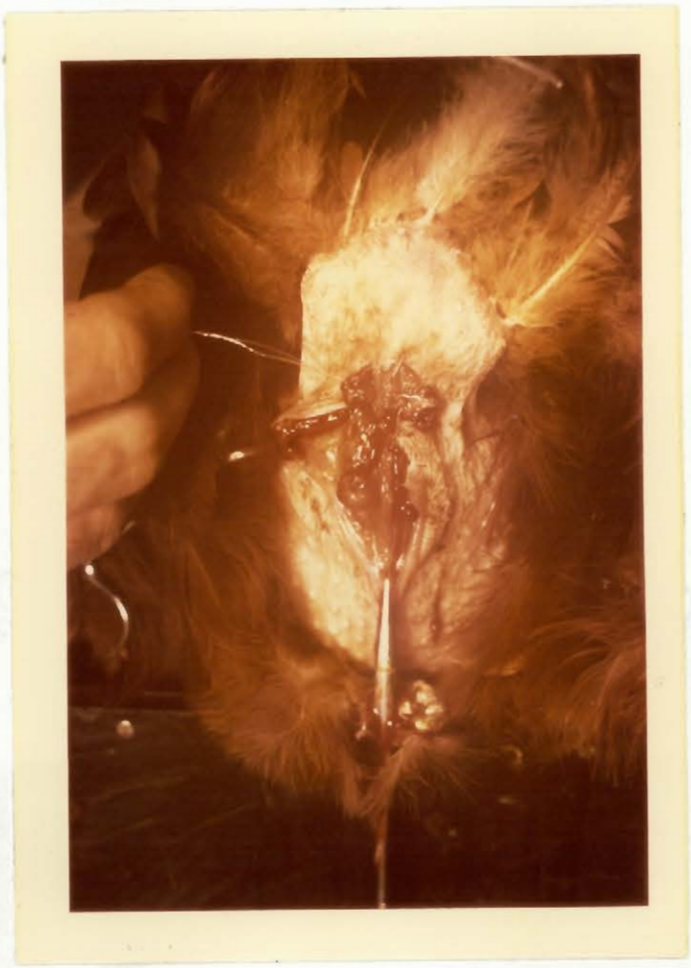


FIGURE 20: Step 3.



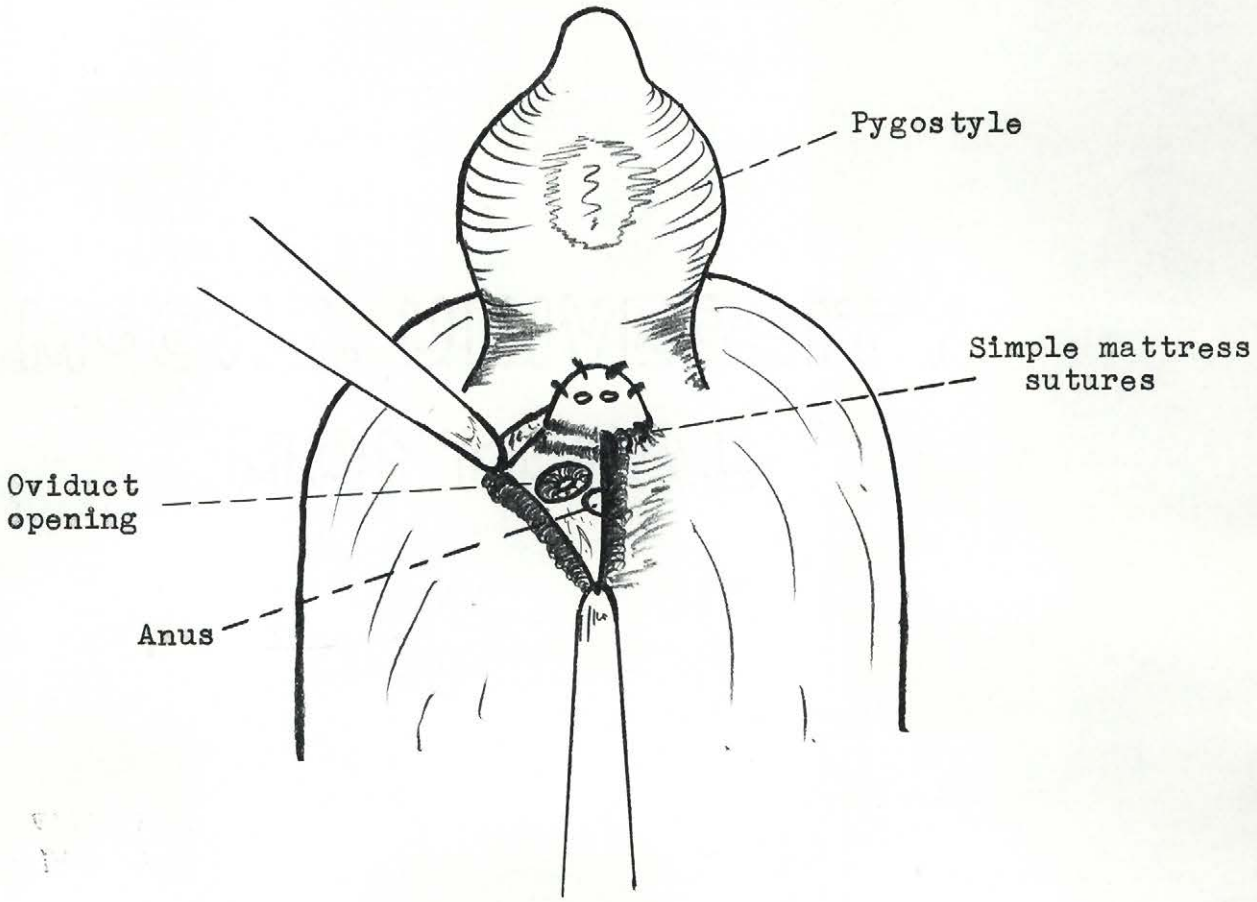


FIGURE 21: Step 4.

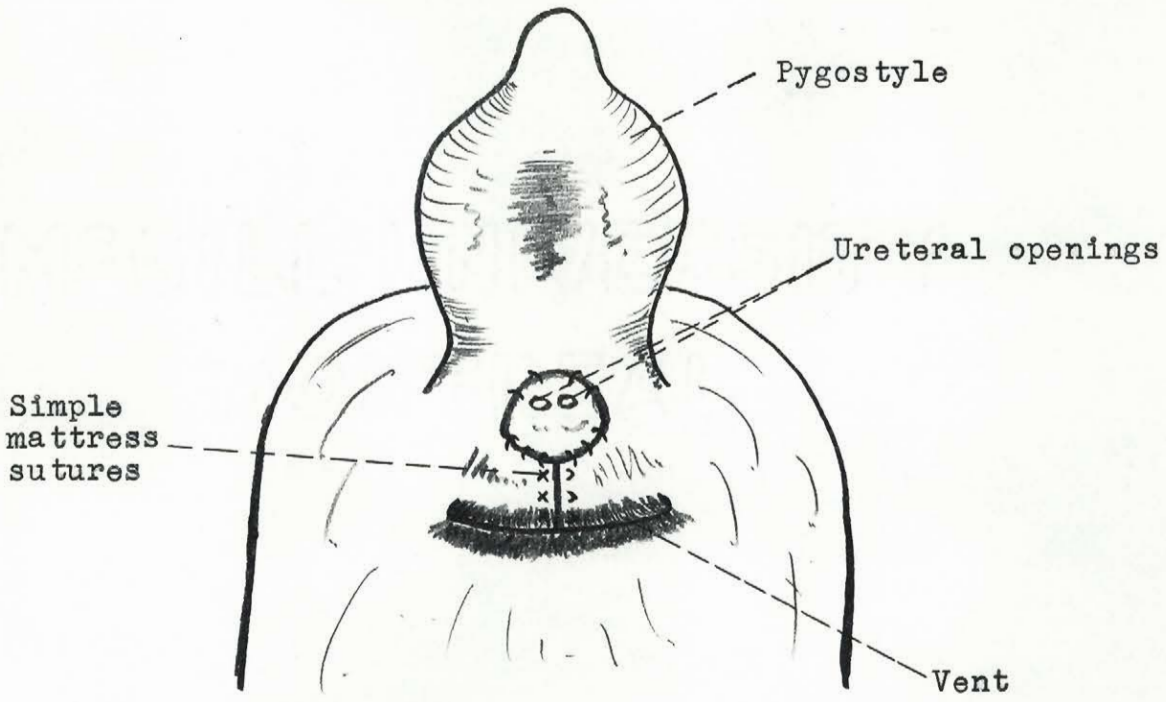


FIGURE 22: Step 4.

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