A STUDY

ON

STEROID HORMONE BIOSYNTHESIS

by

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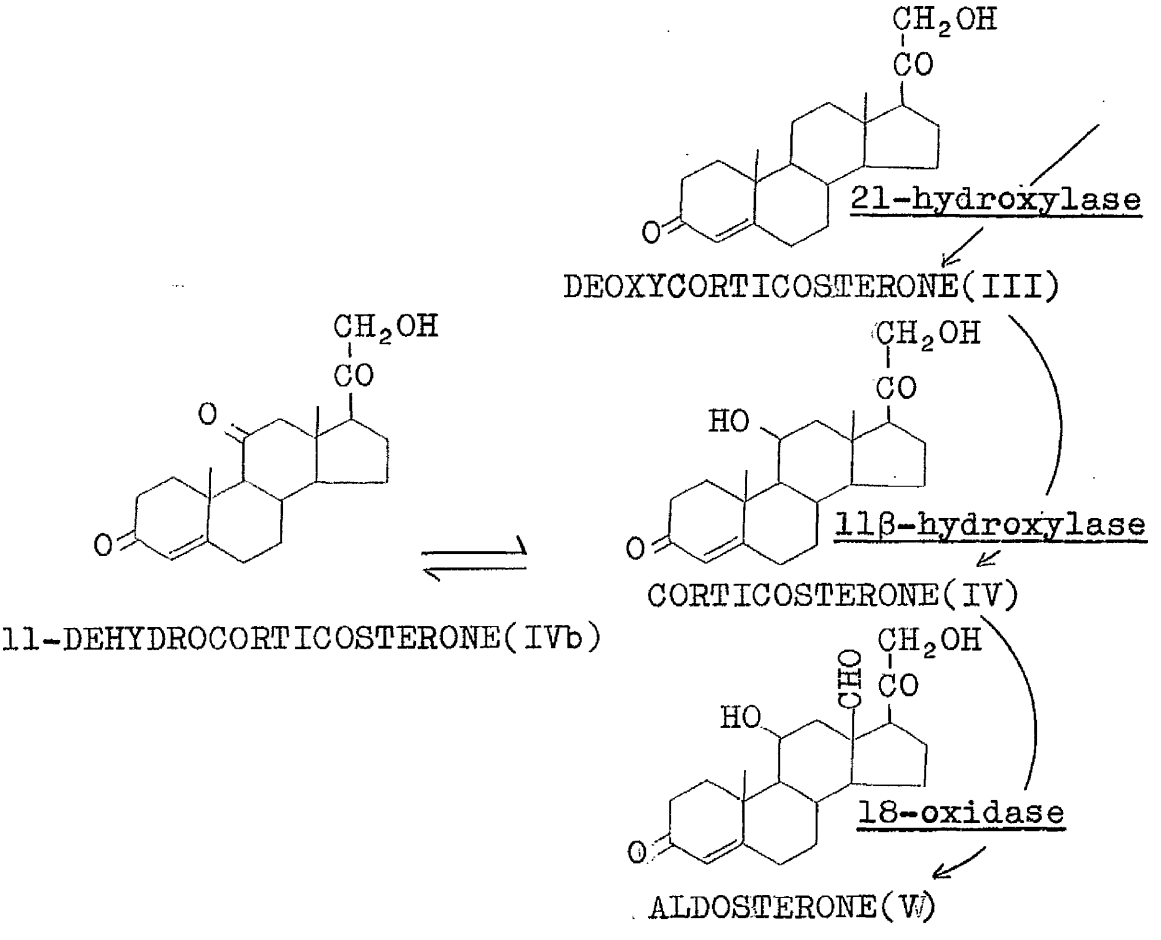
Section I : Introduction

At the outset of this thesis, it should be mentioned that the trivial names of steroids will be used throughout. In Section VIII the steroids are listed in the order of their Roman numeral, giving their chemical nomenclature, the common name used in this thesis and the chemical formula.

Great steps have been made in recent years towards the elucidation of the pathways of steroid hormone biosynthesis. The first step may be said to have been taken by Bloch (1945) when he demonstrated that deuterio cholesterol (I) administered to a pregnant woman gave rise to isotopic pregn~~-4-ene-~~ane-3α:20α-diol (pregnanediol, XXVII) in the urine, assumed to be derived via pregn-4-ene-3:20-dione (progesterone, II). Then in 1951 the first scheme of steroid hormone biosynthesis was published (Hechter et al., 1951). The classical experiments of Hechter and his co-workers, in which the isolated cow adrenal was perfused with possible precursors and intermediates in the pathways of steroidogenesis and the steroid end products isolated and characterised, provided the basis of the now generally accepted scheme which is reproduced in Fig. 1.1.


CH2OH
CO
O
21-hydroxylase
DEOXYCORTICOSTERONE(III)
HO
11β-hydroxylase
CORTICOSTERONE(IV)
CHO
18-oxidase
ALDOSTERONE(V)
11-DEHYDROCORTICOSTERONE(IVb)

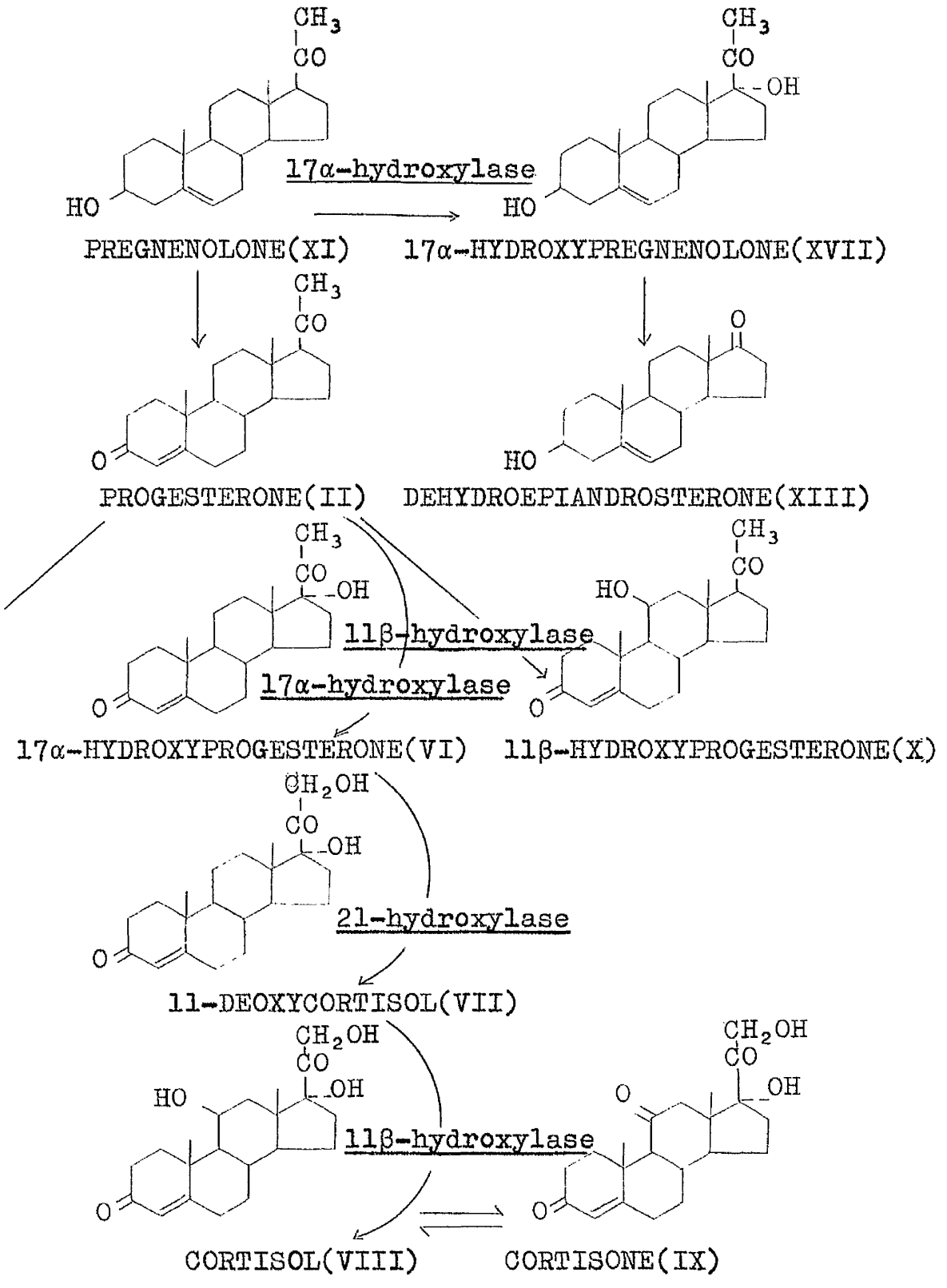


Fig. I.1. Synthesis of corticosteroids in the adrenal cortex.

The present knowledge of the pathways of biosynthesis has been extensively reviewed (Lieberman and Teich, 1953; Dorfman, 1957; Pincus, 1958; Dorfman, 1958; Marrian, 1958; Grant, 1958; Short, 1958; Katzman et al., 1959). In the present discussion it is proposed to deal briefly with the biosynthesis of the corticosteroids, progesterone (II), the androgens and the oestrogens in turn.

The following discussion has been limited to animal transformations. The hydroxylation of the steroid molecule by many microorganisms at several different carbon atoms, as found by Murray and Peterson (1952) has proved of vast importance in the pharmaceutical industry, as well as providing a ready method of studying the mechanism of the reactions. This latter aspect has been reviewed by Eppstein et al. (1956).

From the early work on the biosynthesis of the corticosteroids, by perfusion of the adrenal and incubation of adrenal tissue slices and homogenates with ^{14}C-labelled precursors, it has been shown that the key intermediate is progesterone (II). Several enzyme systems are present in the adrenal to oxidise progesterone (II) at different carbon atoms, yielding the main secretory products of the adrenal. These hydroxylating enzymes are 17α-hydroxylase, 21-hydroxylase, 11β-hydroxylase and 18-oxidase. In Fig. 1.1,

the biosynthesis of the corticosteroids from progesterone (II) is shown. The chemical nomenclature of the steroids shown in the figure is 21-hydroxypregn-4-ene-3:20-dione (deoxycorticosterone, III), 11β:21-dihydroxypregn-4-ene-3:20-dione (corticosterone, IV) 21-hydroxypregn-4-ene-3:11:20-trione (11-dehydrocorticosterone, IVa), 11β:21-dihydroxy-3:20-dioxopregn-4-en-18-al (aldosterone, V), 17α-hydroxypregn-4-ene-3:20-dione (17α-hydroxyprogesterone, VI), 17α:21-dihydroxypregn-4-ene-3:20-dione (11-deoxycortisol, VII), 11β:17α:21-trihydroxypregn-4-ene-3:20-dione (cortisol, VIII), 17α:21-dihydroxypregn-4-ene-3:11:20-trione (cortisone, IX), 11β-hydroxypregn-4-ene-3:20-dione (11β-hydroxyprogesterone, X), 3β-hydroxypregn-5-en-20-one (pregnenolone, XI), 3β-hydroxyandrost-5-en-17-one (dehydroepiandrosterone, XIII) and 3β:17α-dihydroxy-pregn-5-en-20-one (17α-hydroxypregnenolone, XVII). Dorfman (1957), in a very detailed review, tabulates the different systems employed in which acetate, progesterone (II), cholesterol (I) and other compounds give rise to corticosteroids.

The pathway by which progesterone (II) is synthesised in endocrine organs has been investigated. The immediate precursor appears to be pregnenolone (XI) because this compound readily gives rise to progesterone (II) in various adrenal preparations (Hechter *et al.*,

1951; Levy et al., 1954: Samuels, 1953). Staple and his co-workers (1956) isolated pregnenolone (XI) and isocaproic acid arising from the scission of the side chain of cholesterol between carbon atoms 20 and 22, thus confirming and expanding the belief that pregnenolone (XI) is formed by the degradation of the side chain of cholesterol (I) (Hayano et al., 1956; Saba and Hechter, 1955). 20β-Hydroxycholesterol (XII) has been isolated as a radioactive product of the incubation of [4-^{14}C] cholesterol (I) with a bovine adrenal homogenate (Solomon, 1955), an observation which suggests that 20β-hydroxycholesterol (XII) is an intermediate in the pathway between cholesterol (I) and pregnenolone (XI).

The synthesis of cholesterol from acetate has been well established and the mechanisms of the reactions involved have been studied in detail (Bloch, 1958; Ciba Foundation Colloquium, 1959). However several workers have put forth evidence in support of the view that cholesterol is not an obligatory intermediate in the biosynthesis of steroids (Zaffaroni et al., 1951; Hechter et al., 1954; Stone and Hechter, 1954). Perhaps the most striking evidence is that of Heard and his co-workers (1956) who found no radioactivity in cholesterol (I) when a cell-free hog adrenal preparation was incubated with ^{14}C-acetate although cortisol (VIII), corticosterone (IV) and

other corticosteroids showed significant radioactivity. However, although a pathway of steroid biosynthesis has thus been indicated without cholesterol (I) as an intermediate, nothing is known of the relative participation of the two pathways.

In a study of the biosynthesis of the androgens, three endocrine organs must be examined namely the adrenal, the testis and the ovary. That the adrenal synthesises androgens *in vitro* has been established for some years (Kushinsky, 1955: Cooper *et al.*, 1955; Touchstone *et al.*, 1955; Bligh *et al.*, 1955, Bloch *et al.*, 1956a, 1956b; Heard *et al.*, 1956; Rao and Heard, 1957). These conclusions have been confirmed by the presence of the androgens dehydroepiandrosterone (XIII), androst-4-ene-3:17-dione (androstenedione, XIV) and 11β-hydroxyandrost-4-ene-3:17-dione (11β-androstenedione, XV) in adrenal effluent plasma. Although this in itself is not evidence for adrenal androgen synthesis, a comparison of the concentration of these three steroids in peripheral blood and in adrenal blood shows that the latter is much greater, thus indicating secretion of the androgens by the adrenal. The results of the *in vitro* studies with adrenal preparations previously referred to, and of *in vitro* studies with testicular preparations (Brady, 1951; Savard *et al.*,

1952; Wotiz et al., 1955; Lynn, 1956; Lynn and Brown, 1956; Slaunwhite and Samuels, 1956; Savard et al., 1956a, 1956b) indicate that a common pathway of androgen biosynthesis exists in these endocrine tissues. This scheme is shown in Fig. 1.2. There is evidence that the ovary synthesises androgen by the same pathway (Solomon et al., 1956). A more comprehensive discussion of androgen biosynthesis by ovarian tissue will be found in Section III. Dehydroepiandrosterone (XIII), which has not been shown in gonadal tissue, has been shown to arise in a homogenate prepared from human adrenocortical adenoma tissue by a pathway involving pregnenolone (XI) and 17α-hydroxypregnenolone (XVII) with subsequent scission of the side chain to dehydroepiandrosterone (XIII), rather than directly from cholesterol (I) (Goldstein et al., 1958).

In addition androgens may arise from the splitting of the side chain of certain C_{21} steroids. In this way androstenedione (XIV) is the result of the action of a suitable desmolase on 17α-hydroxyprogesterone (VI) or 11-deoxycortisol (VII). Similarly 11β-hydroxyandrostenedione (XV) is formed from cortisol (VIII) and androst-4-ene-3:11:17-trione (adrenosterone, XVIII) from cortisone (IX). This is a peripheral mechanism, demonstrable to only a slight extent and thought to reside mainly in the liver

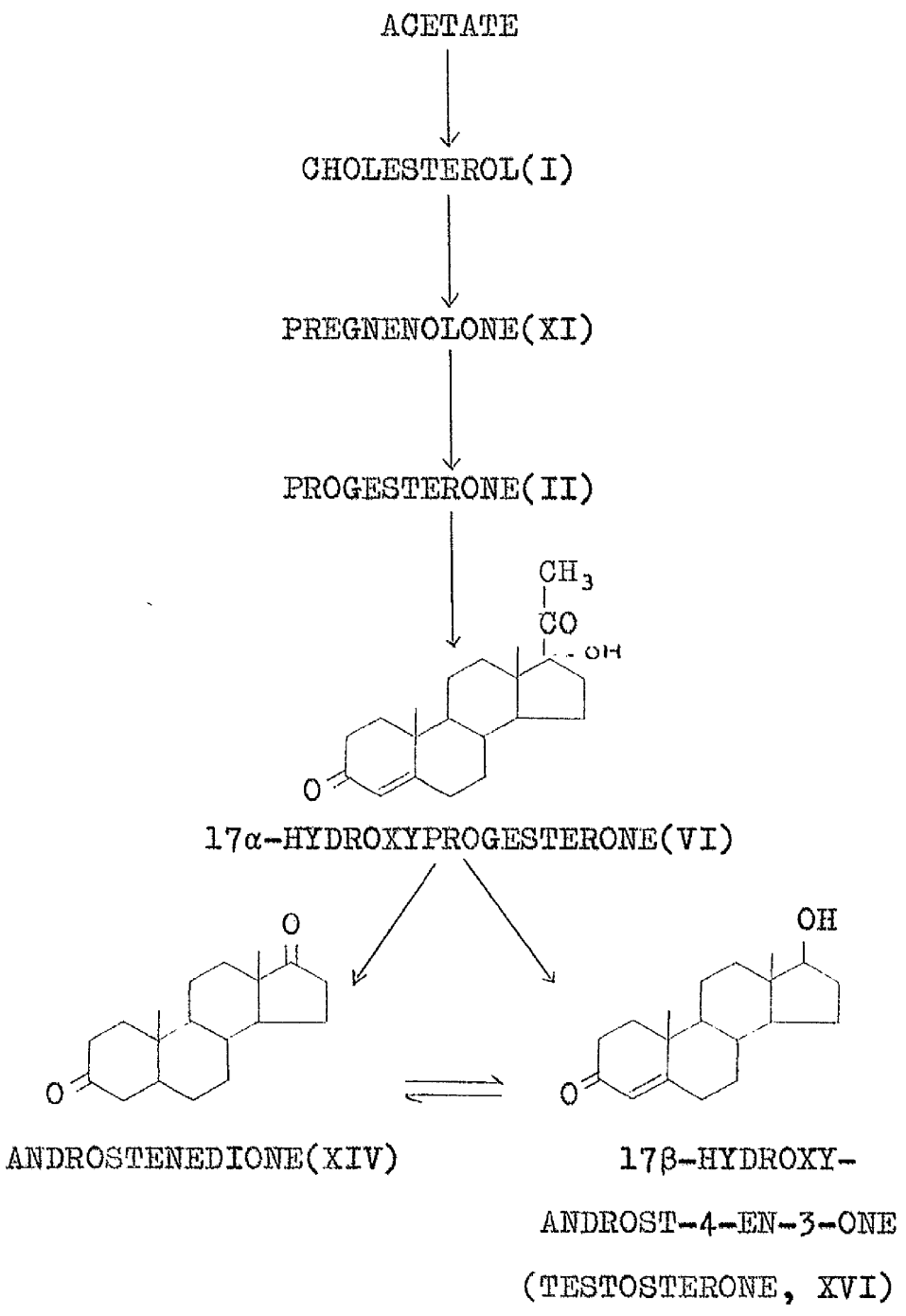


Fig. I.2. Synthesis of androgens in endocrine glands.

(Forchielli et al., 1955 ; Forchielli and Dorfman, 1956).

For some years it has been shown that the C_{19} steroids give rise to oestrogens both in vitro, with ovarian and placental slices (Meyer, 1955; Baggett et al., 1956; Wotiz et al.,1956), and in vivo after the administration of androgens to a pregnant mare (Heard et al., 1955) and to adrenalectomised-ovariectomised women (West et al., 1956). Recently the work of Ryan (1959, 1960) has extended the knowledge regarding the biosynthesis of the oestrogens, 3-hydroxyoestra-1:3:5(10)-trien-17-one (oestrone, XIX), oestra-1:3:5(10)-triene-3:17β-diol (oestradiol-17β, XX) and oestra-1:3:5(10)-triene-3:16α:17β-triol (oestriol, XXI). Ryan's system is a microsomal preparation from human placenta which in the presence of reduced triphosphopyridine nucleotide (TPNH) or a TPNH generating system, converts androstenedione (XIV) and related androgens to oestrogens. By comparing the relative efficiency of the substrates used as precursors of oestrogen, Ryan has obtained results from which he has been able to postulate that aromatisation of the C_{19} steroids proceeds via C-19 hydroxylation. Because 19-nortestosterone (XXII) and androsta-1:4-diene-3:17-dione (XXIII) are converted to oestrogens much less efficiently than 19-hydroxyandrost-4-ene-3:17-dione (19-hydroxyandrostenedione, XXIV) or indeed

than androstenedione (XIV) or testosterone (XVI), Ryan suggests that the C-19 hydroxylation is followed by the introduction of a double bond simultaneously or nearly so with the removal of the angular group at C-19. Complete elucidation of the mechanism of the aromatisation of the C_{19} steroids to yield C_{18} steroids is awaited with great interest.

This thesis presents the results of <u>in vitro</u> incubations of tissue slices of Stein-Leventhal ovaries and of a feminising adrenal tumour. Slices of Stein-Leventhal ovaries were incubated with [1-^{14}C] acetate and the steroids elaborated were studied using the reverse isotope dilution technique. Because of the nature of the Stein-Leventhal syndrome, in which the hirsutism and amenorrhœa suggests an excessive production of androgens, interest was focussed on the ability of the ovaries to synthesise androgens and oestrogens. Evidence will be presented for the biosynthesis of oestrone (XIX), oestradiol (XX), testosterone (XVI), androstenedione (XIV), 17α-hydroxyprogesterone (VI) and progesterone (II) from [1-^{14}C] acetate by tissue slices of Stein-Leventhal ovaries.

Section IV deals with the incubation of slices of a feminising adrenal tumour with [1-^{14}C] acetate and [4-^{14}C] progesterone. Little is known of the capability of this rare type of tumour to synthesise

steroid hormones. Accordingly the steroids elaborated were diluted with oestrogens, androgens, corticosteroids and progesterone (II) to obtain, from a study of these compounds an overall picture of the biosynthetic potential. Evidence will be presented for the synthesis of progesterone (II), cortisol (VIII), androstenedione (XIV) and 17α-hydroxyprogesterone (VI) from [1-^{14}C] acetate by slices of the feminising adrenal tumour. In addition the results indicate the conversion of [4-^{14}C] progesterone to cortisol (VIII), androstenedione (XIV), 17α-hydroxyprogesterone (VI), corticosterone (IV) and 11-deoxycortisol (VII) by slices of the tumour.

Section II : Materials and Methods

Reagents and materials.

Methanol and ethanol were purchased from Burroughs Wellcome Ltd. (AR grade). All other solvents were purchased from British Drug Houses Ltd. (AR grade). All solvents were distilled before use.

Diethyl ether was rendered peroxide-free by being shaken with a saturated aqueous solution of ferrous sulphate and then distilled immediately before use. Peroxide-free ether was used throughout the work being presented.

Glacial acetic acid was purified by being refluxed with potassium permanganate for 30 mins. and then distilled.

Acetic anhydride was purified by distillation from fused sodium acetate.

Pyridine was dried by being refluxed with and then distilled from anhydrous barium oxide. It was stored over sodium hydroxide pellets.

Trimethylacethydrazide ammonium chloride (Girard's Reagent T) was purchased from British Drug Houses Ltd. The reagent was recrystallised from ethanol.

Digitonin was purchased from British Drug Houses Ltd.

Human menopausal gonadotrophin (HMG) was donated by Dr. C. L. Hewett of Organon Laboratories Ltd. and was quoted by them as having an activity of 1 unit per mg.

Adrenocorticotrophic hormone (ACTH) was purchased from Armour Laboratories.

Adenosine triphosphate (ATP) and triphosphopyridine nucleotide (TPN) were purchased from Nutritional Biochemicals Corp., Ohio, U.S.A.

[4-^{14}C] Progesterone (II) and sodium [1-^{14}C] acetate were purchased from the Radiochemical Centre, Amersham, England.

Oestrone (XIX), oestradiol-17β (XX) and oestriol (XXI), androstendione (XIV) and cortisol (VIII) used in the experiments with the feminising tumour of the adrenal, were kindly gifted by Dr. C. L. Hewett of Organon Laboratories Ltd. Androstenedione (XIV), 3α-hydroxy-5α-androstan-17-one (androsterone, XXV), 17α-hydroxyprogesterone (VI), progesterone (II) and testosterone (XVI) which were used in the study of the Stein-Leventhal ovaries were kindly donated by Dr. W. Klyne of the Medical Research Council, London. 17α-Hydroxyprogesterone (VI), corticosterone (IV), and 11-deoxycortisol (VII) which were used in the study of the feminising adrenal tumour were purchased from

Main Research Laboratories, Inc., New York. Dehydroepiandrosterone (XIII) was purchased from Thomson, Skinner and Hamilton Ltd., Glasgow.

Melting-point Determinations

Melting points were determined on a hot stage apparatus (Gallenkamp, England), using the same thermometer throughout. The recorded melting points are uncorrected for the emergent stem.

Assay of Radioactivity

Radioactivity was determined in a windowless gas-flow counter (Tracerlab, Inc.) operating at about 50% efficiency for ^{14}C. The planchets used were copper plates (2.5 cm. diameter) which were cleaned by dipping into chromic acid solution for a few seconds, followed by thorough washing in tap water, distilled water, alcohol and ether. The plates were finally dried in air. Samples of material to be assayed were dispensed from methanolic solutions. Plates were counted at infinite thinness for a sufficient length of time to give a counting error of less than 5%.

Specific activities throughout are expressed as counts per minute (cpm) per mg. Where derivatives have been formed, specific activities have been calculated as cpm per mg. free compound.

Concentration of Solutions.

Removal of solvents from extracts was carried out by distillation in vacuo on a water bath at a temperature not exceeding 45°.

Small volumes of solvents from extracts were evaporated under a stream of nitrogen, on a water bath at temperatures below 45°.

Paper Chromatography.

Before chromatography Whatman No. 1 chromatography paper was washed by allowing methanol to flow down it for 24 hours in a chromatography tank. The paper was dried in air.

The toluene-propylene glycol solvent system (Zaffaroni and Burton, 1951) and the ligroin-propylene glycol solvent system (Savard, 1953) were used to separate mixtures of steroids on paper. Androstenedione (XIV), 17α-hydroxyprogesterone (VI), testosterone (XVI), progesterone (II), cortisol (VIII), corticosterone (IV), 11-deoxycortisol (VII) and deoxycorticosterone (III) were detected on paper by their absorption of uv light (maximum ~~trans~~emission, 253.7 mμ). Androsterone (XXV), dehydroepiandrosterone (XIII) and oestrone (XIX) were detected by the modification of the Zimmermann reaction described by Savard (1953). The Folin-Ciocalteau

reagent as used by Layne and Marrian (1958) detected oestradiol (XX) and oestriol (XXI).

Steroids were eluted from the paper with methanol (5-10 ml.).

Measurement of Steroids.

The concentration of a methanolic solution of a Δ^4-3 ketosteroid was determined from the absorption maximum at or about 240 mμ, that of an oestrogen from the maximum at or about 280 mμ and that of the oestrogen acetates from the maxima at 267 mμ and 275 mμ. The concentration is calculated from the equation

$$\varepsilon c = K$$

where ε is the molar extinction coefficient of the solute for the particular wavelength in question, c is the concentration of the solute in g. moles per litre and K is the extinction coefficient, equal to the optical density when the light path of the solution is 1 cm. Optical densities were measured using a spectrophotometer (Unicam, Model SP. 500) and cells of 1 cm. light path. The following values were determined with solutions of authentic samples in methanol.

Steroid	λ_{max}^{MeOH} (mμ)	ε
oestrone (XIX)	280	2129
oestrone monoacetate	1. 268	759
	2. 275	704
oestradiol (XX)	280	2047
oestriol (XXI)	280	2158
oestriol triacetate	1. 268	842
	2. 275	761
cortisol (VIII)	240	15900
cortisol 21-monoacetate	240	16900
11-deoxycortisol (VII)	240	15700
deoxycorticosterone (III)	240	17410
corticosterone (IV)	240	15600
progesterone (II)	240	16500
17α-hydroxyprogesterone (VI)	240	16400
androstenedione (XIV)	240	15700
testosterone (XVI)	240	15600
testosterone monoacetate	240	19700

Dehydroepiandrosterone (XIII) was estimated by the method of Oertel and Eik-Nes (1959).

Preparation of Acetates.

When less than 1 mg. material was available for acetylation, the material was dissolved in 0.4 ml. dry pyridine and 0.2 ml. acetic anhydride was added.

The reaction was allowed to proceed at room temperature for 18 hours, then stopped by the addition of a few drops of methanol. The reaction mixture was evaporated to dryness under nitrogen.

The same procedure, using 2 volumes pyridine to 1 volume acetic anhydride was followed when more than 1 mg. material was available for acetylation. The reaction was stopped by adding the reaction mixture dropwise to water (50 ml.). The acetate derivative was extracted with ether (2 x 50 ml., 2 x 25 ml.) and the ether phase washed with N HCl (3 x 1/10 vol.), water (3 x 1/10 vol.), 5% (w/v) Na_2CO_3 (3 x 1/10 vol.) and water to neutrality. The ethereal solution of the acetate was evaporated to dryness.

Hydrolysis of oestradiol diacetate.

Oestradiol diacetate was hydrolysed by refluxing with 5% (w/v) KOH in 80% (v/v) ethanol (1 ml. per mg. oestradiol diacetate) for 2 hours. After acidification, the oestradiol (XX) was extracted with ether. The ether was washed with water till no longer acid and evaporated to dryness.

Preparation of androstenedione-bis-dinitrophenylhydrazone.

Androstenedione-bis-dinitrophenylhydrazone was prepared by Method A of Reich et al., (1953).

In this method excess dinitrophenylhydrazine is discharged by the addition of Benedict's reagent and the steroid-DNP extracted into chloroform. The residue obtained by evaporation of the chloroform extract was further purified on a column of silica gel, the androstenedione-bis-DNP being eluted from the column partially by benzene and totally by 1% (v/v) ethyl acetate in benzene.

Hydrolysis of androstenedione-bis-DNP.

The androstenedione-bis-DNP (9 mg.) was dissolved in chloroform (4 ml.). Concentrated HCl (3 ml.) was added together with sufficient ethanol (6 ml.) to produce a homogeneous mixture. The mixture was refluxed for 5 hours, then diluted with water (100 ml.). The product was extracted with ether and the ether solution washed with 2N HCl (3 x 1/10 vol.), then water till no longer acid. The ethereal solution was evaporated to dryness and the androstenedione was purified by paper chromatography.

Sulphuric acid Chromogens.

To thoroughly dried steroid (10-60 µg.) 3 ml. concentrated H_2SO_4 was added. After 2 hours at room temperature, the spectrum of the sulphuric acid chromogen was read over the range 200 mµ to 600 mµ. For

reference, the spectra of the sulphuric acid chromogens of several authentic steroids are reported in Table II. 1. The notation is that of Bernstein and Lenhard (1953). An absorption band is designated by a single wavelength. The symbol [I] denotes an inflection or plateau, represented by a single wavelength. The same system is used throughout this thesis.

Table II. 1. Sulphuric acid chromogen maxima and minima of authentic steroids.

STEROID	CONCN. in 3 ml. conc. H_2SO_4	MAXIMA mμ (O.D.)	MINIMA mμ (O.D.)
oestrone (XIX)	48.5 μg.	232(.550) [I]; 300(.844); 446(.578).	253(.146); 370(.041).
oestrone acetate	104 μg. (acetate)	232(.712) [I]; 300(1.038); 448(.695).	252(.193); 365(.048).
oestradiol (XX)	51 μg.	278(.291); 310(.310); 367(1.005); 424(.958); 442(.562) [I].	260(.244); 288(.247); 318(.300); 390(.570).
oestriol (XXI)	40 μg.	231(.342) [I]; 305(.215); 452(.146).	263(.050); 331(.040).
oestriol triacetate	91 μg. (acetate)	232(.460) [I]; 305(.301); 354(.108); 449(.110).	265(.116); 332(.092); 393(.070).
cortisol (VIII)	41 μg.	232(.555) [I]; 278(.560); 392(.278); 468(.355).	250(.375); 340(.137); 412(.230).
cortisol 21-mono-acetate	9.2 μg. (acetate)	234(.121) [I]; 284(.170); 392(.065).	253(.095); 360(.053).
11-deoxycortisol 21-mono-acetate	17 μg. (acetate)	218(.142); 238(.115) [I]; 288(.259); 332(.090) [I]; 538(.060).	248(.105); 420(.025).

Table II. 1. (contd.)

STEROID	CONCN. in 3 ml. conc. H_2SO_4	MAXIMA mμ (O.D.)	MINIMA mμ (O.D.)
corticosterone 21-mono-acetate	61 μg. (acetate)	288(.870); 335(.251); 376(.156); 474(.169).	233(.289); 322(.239); 366(.146); 406(.105).
progesterone (II)	51 μg.	223(.365); 292(.960).	233(.345).
17 α -hydroxy-progesterone (VI)	48 μg.	246(.302) [I]; 288(.860); 429(.510).	233(.285); 335(.044).
androstenedione (XIV)	49.2 μg.	296(1.42).	237(.167).

Section III : Incubation of Stein-Leventhal ovaries with sodium [1-^{14}C] acetate

I. Introduction.

The syndrome of large polycystic ovaries with thickened capsules, associated with hirsutism, amenorrhœa and sterility was first described by Stein and Leventhal in 1935.

Opinions differ as to the origin of the androgens bringing about the hirsutism in this syndrome. In many cases elevated urinary androsterone (XXV) and 3α-hydroxy-5β-androstan-17-one (etiocholanolone, XXVI) with normal or slightly elevated dehydroepiandrosterone (XIII) have been found. It is reported that these values are not significantly depressed on the administration of cortisone (Johnsen, 1956; Pesonen and Mikkonen, 1958). On the other hand Perloff (1957) and Gallagher and his co-workers (1958) maintain that the pattern of the urinary 17-ketosteroids is similar to that of idiopathic hirsutism in which excessive adrenal production of 11-deoxysteroids is indicated. The levels are increased by ACTH and suppressed by corticosteroid in these cases. These observations certainly suggest the involvement of some precursor(s) arising from the adrenal cortex. However, they do not explain the relief of the symptoms given by

bilateral wedge resection of the ovaries. It is interesting to note the classification of Stein-Levanthal ovaries by Pesonen et al., (1959). These authors list two main types, the first in which the ovaries are active although enlarged, and which they suggest is caused by a defect predominantly of ovarian origin, and the other in which there is an absence of signs of ovarian function and which is best interpreted as the result of the disorder of a gland other than the ovary, for example, the adrenal gland. Lanthier (1960) apparently feels any such classification rather artificial, preferring to view the differing clinical findings as manifestations, in different degrees, of the same disorder of steroid metabolism, in which both the ovary and the adrenal are concerned.

Indirect evidence of androgen production by the ovary was given by Katsh (1950) who demonstrated androgenic activity of ovarian transplants to the seminal vesicle of castrated male rats. Further evidence came from the work of Solomon et al., (1956) who showed the conversion of progesterone (II) to 17α-hydroxyprogesterone (VI) and androstenedione (XIV) by a bovine ovarian homogenate. The isolation of these two steroids from extracts of human ovaries (Zander, 1958) has strengthened the theory that the ovary is capable of synthesising androgens.

Several workers have reported the synthesis

of oestrogens by ovarian tissue *in vitro*. The conversion by human ovarian tissue slices of [3-^{14}C] testosterone to ^{14}C-oestradiol-17β (Baggett *et al.*, 1956) and to ^{14}C-oestradiol-17β, ^{14}C-oestrone and ^{14}C-oestriol (Wotiz *et al.*, 1956) has been demonstrated. The conversion of ^{14}C-acetate to oestrone and oestradiol-17β has been shown in ovarian systems both by a dog ovarian homogenate (Rabinowitz and Dowben, 1953) and by perfusion of sow ovaries (Werthessen, 1953).

The following work presents evidence for the synthesis of progesterone, oestrogens and androgens from sodium [1-^{14}C] acetate by *in vitro* incubations of Stein-Levanthal ovaries.

2a. Incubation Conditions.

Ovarian tissue was obtained from women with the Stein-Levanthal syndrome undergoing bilateral wedge resection of the ovaries. About an hour elapsed between removal of the tissue and the start of the incubation period. The tissue was sliced by hand with a Stadie-Riggs microtome and incubated for 2-1/2 hours at 37° C with continuous shaking in an atmosphere of oxygen in Krebs-Ringer phosphate buffer (pH 7.4) containing sodium [1-^{14}C] acetate (0.01 M, specific activity 2 μc/mg.), glucose (0.01 M), nicotinamide (0.04 M) and fumarate (0.01 M). In two incubations human menopausal gonadotrophin (HMG, 1 unit per mg.) was added. The incubations were

I. (M.O'D): 2.7 g. slices in 20 ml. buffer with 5 mg. HMG.

IIa. (H.McA. left side): 5.31 g. slices in 15 ml. buffer with 5 mg. HMG.

IIb. (H.McA. right side): 5.22 g. slices in 15 ml. buffer without HMG.

2b. Extraction.

The procedure was identical in all three incubations. The tissue and the incubation medium were separated and the incubation medium was extracted with ethyl acetate (6 x equal volume). The tissue was

homogenised in water (20 ml.) and this homogenate extracted with ethyl acetate (6 x 20 ml.). The ethyl acetate extracts were combined and evaporated to dryness.

The crude extract was dissolved in heptane (30 ml.), and extracted 4 times with 90% aqueous methanol (60 ml. portions). The heptane fraction and the aqueous methanol fractions were taken to dryness and assayed for radioactivity.

β-Sterol Fraction

The β-sterol fraction (mainly cholesterol) of the heptane phase was precipitated as the digitonide. A solution of digitonin (5 x weight of the heptane fraction, concentration of 10 mg./ml.) in hot 80% (v/v) aqueous ethanol was added to the heptane fraction dissolved in hot ethanol (10 mg./ml.). The digitonide formed was washed with acetone:ether (1:1, v/v) and then with ether.

The digitonide was cleaved to give a β-sterol fraction (cholesterol) by heating in dry pyridine at 70-80° C for 30 minutes (Bergmann, 1940). The pyridine was evaporated and the cholesterol isolated by extracting the residue with ether. The specific activity of the cholesterol was determined.

Phenolic Extraction.

The residue from the 90% methanol fraction was dissolved in toluene (100 ml.). The toluene solution was extracted 5 times with N NaOH (25 ml. portions), then with water to neutrality and evaporated to dryness. This residue contained the neutral steroids.

The combined alkali and aqueous phases were acidified with conc. HCl and extracted 6 times with ether (50 ml. portions). The combined ether extracts were washed with water to neutrality and evaporated to dryness. The residue obtained contained the phenolic steroids.

Separation of an 'Oestriol' Fraction

The phenolic residue was separated into an 'oestrone-oestradiol' fraction and an 'oestriol' fraction by the method of Friedgood et al., (1948). The phenolic fraction dissolved in benzene (100 ml.) was extracted 3 times with a solution of 0.075 M Na_2HPO_4 (50 ml. portions). The benzene residue (the 'oestrone-oestradiol' fraction) was evaporated to dryness.

The aqueous phase was taken to pH 2 by the addition of dilute H_2SO_4 and extracted 4 times with ether (75 ml. portions). The ethereal extract was washed with water to neutrality and evaporated to dryness. The material obtained was the 'oestriol' fraction.

Separation of an 'oestrone' fraction.

An 'oestrone' fraction was obtained by the micro-Girard separation described by Pincus and Pearlman (1941). Glacial acetic acid (2 ml.) and Girard's Reagent T (400 mg.) were added to the 'oestrone-oestradiol' fraction and the reaction mixture was refluxed for 30 minutes under anhydrous conditions. After cooling, the contents were added to ice-cold 0.45 N NaOH (75 ml.) and the pH adjusted to between 6.7 and 7.0. The mixture was extracted 4 times with ether (50 ml. portions). The ether was washed with ice-cold water (20 ml.), 2.5% (w/v) Na_2CO_3 (20 ml.) and water to neutrality. The initial water wash was added to the aqueous phase, the other washes being discarded. The ether, containing the non-ketonic 'oestradiol' fraction, was evaporated to dryness.

The aqueous phase was adjusted to pH 1 with concentrated HCl and allowed to hydrolyse at room temperature for 2 hours, and extracted 4 times with ether (50 ml. portions). The ether phase after being washed with 2.5% (w/v) Na_2CO_3 (20 ml.) and water to neutrality, was evaporated to dryness. The material obtained was the ketonic 'oestrone' fraction.

A summary of the extraction procedure is given schematically in Fig. III.1. At each stage of the extraction, the radioactivity assays were done.

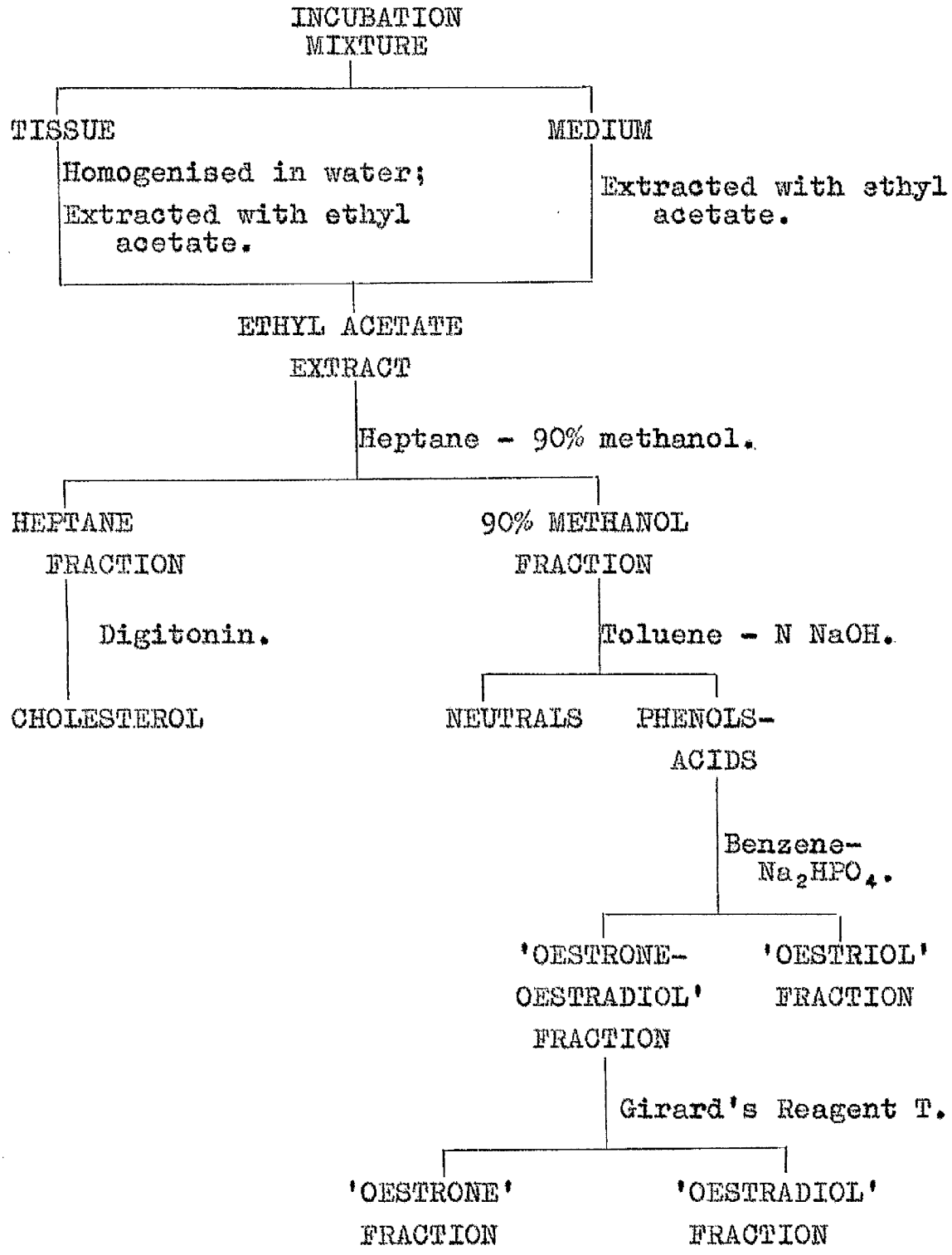


Fig. III.1. Extraction and Purification Procedure.

The Oestrogens.

The oestrogen fractions were each diluted with the respective carrier and purified by repeated crystallisations. The melting point and the specific activity of each crop of crystals were determined. Unfortunately the 'oestradiol' and 'oestriol' fractions of Experiment I were lost. The amounts of carrier added are shown in Table III.1.

In experiment IIa it was found that two crystallisations from aqueous methanol of the oestradiol-17β (XX) separated the radioactivity from the crystalline material. The mother liquor from the first crystallisation was then acetylated, the product being crystallised twice. The oestradiol diacetate was hydrolysed and the product crystallised three times from aqueous methanol. In Experiment IIb after addition of carrier oestradiol-17β (XX) to the 'oestradiol' fraction, the mixture was immediately acetylated, the product being crystallised, then hydrolysed. The free oestradiol-17β (XX) was crystallised from aqueous methanol.

The Neutral Steroids.

The neutral fractions obtained from experiments IIa and IIb were chromatographed on paper for 48 hours (solvent system, ligroin-propylene glycol). To

Table III. 1. Addition of the respective carrier to each radioactive oestrogen fraction.

Experiment	I	IIa	IIb
'Oestrone'			
Cpm.	1700	280	130
Wt. carrier oestrone (mg.)	8.24	6.20	6.08
'Oestradiol'			
Cpm.	-	6075	1300
Wt. carrier oestradiol-17β (mg.)		25.95	10.85
'Oestriol'			
Cpm.	-	970	1350
Wt. carrier oestriol (mg.)		5.10	5.00

the overflow a mixture of androsterone (XXV, 56 μg.), androstenedione (XIV, 49 μg.) and progesterone (II, 46.5 μg.) was added. These steroids were resolved by paper chromatography in the solvent system ligroin-propylene glycol. The original chromatogram was eluted and a mixture of testosterone (XVI, 54.5 μg.) and 17α-hydroxyprogesterone (VI, 52.4 μg.) was added to the eluate. However these steroids do not separate in the ligroin-propylene glycol system.

Additional carrier material was added to each steroid fraction in the following amounts: androsterone (XXV, 305 μg.), androstenedione, (XIV, 350 μg.), progesterone (II, 279 μg.), testosterone (XVI, 182 μg.) and 17α-hydroxyprogesterone (VI, 237 μg.). The mixture of testosterone (XVI) and 17α-hydroxyprogesterone (VI) was separated after acetylation, testosterone acetate having a much greater mobility than 17α-hydroxyprogesterone (VI) in the ligroin-propylene glycol solvent system. Each steroid was purified by successive chromatography on paper, the specific activities of the eluted materials being measured at each stage.

3. Results

At every stage of the extraction procedure the radioactivity in each fraction was assayed. The results obtained, expressed as cpm and as percentage incorporation of the ^{14}C-acetate precursor, are shown in Table III.2. It is evident that there was considerable variation from one experiment to another.

Cholesterol (I)

The cholesterol isolated from each experiment was found to be radioactive. In Experiment I the heptane fraction of specific activity 950 cpm per mg. yielded cholesterol, specific activity 1190 cpm per mg. The heptane fraction, specific activity 9400 cpm per mg., of the Experiment IIa gave cholesterol of specific activity 10,000 cpm per mg. The heptane fraction, specific activity 7200 cpm per mg., of Experiment IIb yielded cholesterol of specific activity 12,000 cpm per mg.

Oestrone (XIX)

Experiment I

8.24 mg. of oestrone was added to the 'oestrone' fraction (1700 cpm) giving a total weight of 9.01 mg. of material of specific activity 189 cpm per mg. The material was recrystallised twice from

Table III. 2. Counts per minute and percentage incorporation of sodium [1-^{14}C] acetate in each fraction of the extraction procedure.

Experiment	I		IIa		IIb	
Sodium [1-^{14}C] acetate incubated.	3.6 x 10^7 cpm		3.9 x 10^7 cpm		3.9 x 10^7 cpm	
FRACTION:	Cpm	%age conversion	Cpm	%age conversion	Cpm	%age conversion
Heptane	9500	0.027	76400	0.20	81200	0.21
90% methanol	10.60 x 10^6	29.7	2.25 x 10^6	5.8	1.79 x 10^6	4.6
Neutrals	18500	0.052	15800	0.41	111000	0.28
Phenols-Acids	42000	0.118	7000	0.018	8900	0.023
'Oestriol'	31500	0.088	970	0.0025	1350	0.003
'Oestrone-Oestradiol'	7500	0.021	7770	0.020	1850	0.005
'Oestrone'	1700	0.005	280	0.0007	130	0.0003
'Oestradiol'	4900	0.014	6075	0.016	1300	0.003

aqueous methanol. The results obtained from melting point determinations of crystals and specific activity determinations of crystals and mother liquor are shown in Table III.3. The final material, m.p. 254-256°, had a specific activity of 132 cpm per mg. This represents a minimum incorporation of [1-^{14}C] acetate of 0.003% into oestrone.

Experiment IIa

6.20 mg. of oestrone was added to the 'oestrone' fraction (280 cpm) giving a total weight of 6.82 mg. of material, specific activity 41 cpm per mg. Crystallisation from aqueous methanol yielded 5.42 mg. of crystals (m.p. 248-253°), specific activity 33 cpm per mg. This represents a minimum of 0.0006% incorporation of [1-^{14}C] acetate into oestrone.

Experiment IIb.

6.08 mg. of carrier was added to the 'oestrone' fraction (130 cpm) giving 6.30 mg. of material, specific activity 20.6 cpm per mg. Crystallisation from aqueous methanol yielded 5.10 mg. of crystals (m.p. 247-253°) of specific activity 19.6. The minimum incorporation of [1-^{14}C] acetate into oestrone was 0.0003%.

Table III. 3. Crystallisation of oestrone in Experiment I : Specific activities (sp.act.) of crystals and mother liquors.

	CRYSTALS			MOTHER LIQUOR	
Crystallisation	Wt. (mg.)	M.p.	Sp.act. (cpm/mg.)	Wt. (mg.)	Sp.act. (cpm/mg.)
1	5.31	238-244°	120	2.63	278
2	4.65	254-256°	132	0.76	175

Oestradiol-17β (XX)

Experiment IIa

25.95 mg. of oestradiol carrier was added to the crude fraction (6075 cpm) giving a total weight of 29.10 mg., specific activity 209 cpm per mg. Two crystallisations separated the crystals from the radioactivity. The results are shown in Table III.4.

The mother liquor from the first crystallisation was acetylated and the product crystallised twice from aqueous methanol, then hydrolysed. The reaction product was crystallised three times, finally yielding material (m.p. 168-172°) of specific activity 826 cpm per mg. If the possibility of additional ^{14}C-oestradiol diacetate being present in the mother liquor of the second crystallisation of oestradiol-17β (i.e. before acetylation) is ignored, this represents a minimum of 0.014% conversion of [1-^{14}C] acetate to ^{14}C-oestradiol or ^{14}C-oestradiol diacetate. In the results summarised in Table III.5, the specific activities of the diacetate have been calculated as cpm per mg. of free oestradiol-17β.

Experiment IIb.

10.85 mg. oestradiol carrier was added to the 'oestradiol' fraction (1300 cpm) giving a total weight of 11.70 mg. of material, specific activity 108 cpm

Table III. 4. Crystallisation of oestradiol-17β in Experiment IIa : Specific activities (sp.act.) of crystals.

	CRYSTALS		
Crystallisation	Wt. (mg.)	M.p.	Sp.act. (cpm/mg.)
1	22.00	170-172°	39.6
2	15.43	174-177°	3.6

Table III. 5. Acetylation of oestradiol-17β and subsequent hydrolysis in Experiment IIa : Specific activities (sp.act.) of products and crystals are calculated as cpm. per mg. free oestradiol-17β in every instance.

	Wt. (mg.)	M.p.	Sp.act. (cpm/mg.)
Mother liquor	6.31	-	830
Acetylation Product	6.55	-	950
Crystallisation : 1	5.45	96-108°	638
2	4.70	115-117°	668
Hydrolysis Product	3.80	-	680
Crystallisation : 1	2.50	168-172°	778
2	1.70	168-172°	804
3	1.15	168-172°	826

per mg. This material was acetylated and the product was crystallised from aqueous methanol, then hydrolysed. Crystallisation of the hydrolysis product from aqueous methanol yielded crystals of specific activity 89 cpm per mg. This indicates a minimum of 0.003% incorporation of [1-^{14}C] acetate. The results are shown in Table III.6.

Oestriol (XXI)

Experiment IIa.

5.10 mg. of oestriol was added to the crude 'oestriol' fraction (970 cpm) giving 5.48 mg. of material, specific activity 190 cpm per mg. Two crystallisations were performed from aqueous methanol but no constancy in specific activity was achieved. The results are shown in Table III.7.

Experiment IIb.

5.00 mg. of oestriol carrier was added to the 'oestriol' fraction (1350 cpm) giving a total weight of 5.55 mg. of material, specific activity 242 cpm per mg. Crystallisation yielded 1.05 mg. of crystals (m.p. 260-264°) of specific activity 83 cpm per mg.

Table III. 6. Acetylation of oestradiol-17β and subsequent hydrolysis in Experiment IIb : Specific activities (sp.act.) are calculated as cpm per mg. free oestradiol-17β.

	Wt. (mg.)	M.p.	Sp.act. (cpm/mg.)
Acetylation Product	13.70	-	115
Crystallisation	12.50	119-121°	79
Hydrolysis and Crystallisation	3.70	168-172°	89

Table III. 7. Crystallisation of oestriol in Experiment IIa : Specific activities (sp.act.) of crystals.

Crystallisation	Wt. (mg.)	M.p.	Sp.act. (cpm/mg.)
1	3.09	264-266°	47
2	1.80	265-268°	22

Neutral Steroids

After dilution with carrier, the steroids being studied, androsterone (XXV), androstenedione (XIV), progesterone (II), testosterone (XVI) and 17α-hydroxy-progesterone (VI) were further purified by chromatography on paper (solvent system, ligroin-propylene glycol). Androsterone (XXV) proved to be only slightly radioactive and its study was not pursued. Androstenedione (XIV), after three chromatograms, appeared to have constant specific activity (934 cpm per mg.) in Experiment IIa but no constancy was achieved in Experiment IIb. After five chromatograms the specific activity of progesterone (II) was constant (1251 cpm per mg.) in Experiment IIa but not so in IIb. The specific activity of testosterone (XVI) was constant in Experiment IIa after two paper chromatograms, 3034 cpm per mg. (calculated as free testosterone) and in Experiment IIb after four chromatograms, 1516 cpm per mg. (calculated as free testosterone). 17α-Hydroxyprogesterone (VI) had constant specific activity in both experiments after two chromatograms, 2640 cpm per mg. in Experiment IIa and 1028 cpm per mg. in Experiment IIb. The specific activities of these steroids eluted from each chromatogram together with the percentage minimum incorporation of $[1\text{-}^{14}C]$ acetate into each steroid are given in Tables III.8-III.11 inclusive.

Table III. 8. Specific activities (sp.act.) of androstenedione from successive paper chromatograms.

Chromatogram	Sp.act.(cpm/mg.) of androstenedione Experiment IIa	Experiment IIb
1	1055	1370
2	498	1030
3	367	934
Minimum incorporation of [1-^{14}C] acetate	0.0004%	0.0010%

Table III. 9. Specific activities (sp.act.) of progesterone from successive paper chromatograms.

Chromatogram	Sp.act.(cpm/mg.) of progesterone Experiment IIa	Experiment IIb
1	2610	7280
2	2410	1110
3	1360	229
4	1229	437
5	1251	312
Minimum incorporation of [1-^{14}C] acetate	0.0011%	0.0003%

Table III. 10. Specific activities (sp.act.) of testosterone from successive paper chromatograms. The specific activities have been calculated as cpm. per mg. testosterone.

	Sp.act.(cpm/mg.) of testosterone	
Chromatogram	Experiment IIa	Experiment IIb
1	3160	895
2	3034	643
3	-	1378
4	-	1516
Minimum incorporation of [1-^{14}C] acetate	0.0020%	0.0010%

Table III. 11. Specific activities (sp.act.) of 17α-hydroxyprogesterone from successive paper chromatograms.

	Sp.act.(cpm/mg.) of 17α-hydroxyprogesterone	
Chromatogram	Experiment IIa	Experiment IIb
1	2750	879
2	2640	1028
Minimum incorporation of [1-^{14}C] acetate	0.0021%	0.0008%

4. Discussion

The criterion of radiochemical purity of the steroids studied was constancy of specific activity of the crystalline compounds through two or more crystallisations or constancy of specific activity of the neutral steroids through two or more paper chromatograms. On this basis it has been shown that oestrone (XIX) has been synthesised from sodium [1-^{14}C] acetate in Experiments I and IIb. The evidence in Experiment IIa is not conclusive. In Experiments IIa and IIb, radioactive oestradiol acetate was found. From a comparison of the specific activities of the acetate with those of the hydrolysed product, it is apparent that the acetate moieties are not themselves radioactive. Thus it is likely that the acetylation has occurred during the extraction procedure. The only probable stage where this might happen is the micro-Girard separation of 'oestrone' and 'oestradiol' fractions. If trace amounts of acetic anhydride were present in the glacial acetic acid used, acetylation may have taken place. Radioactive oestriol (XXI) was not found in any of the three experiments.

Testosterone (XVI) and 17α-hydroxyprogesterone (VI) in Experiments IIa and IIb, androstenedione (XIV) in Experiment IIb and progesterone (II) in Experiment IIa were adjudged to be radioactive by the criterion defined above. Study of these compounds

was limited by the recovery both of material and of radioactivity from each successive paper chromatogram. It is possible that on the paper chromatograms other compounds, possibly radioactive, may have the same mobilities as androstenedione (XIV) and progesterone (II) respectively, in the ligroin-propylene glycol solvent system used. The same criticism may be held against the evidence for the radiochemical purity of the testosterone (XVI), and 17α-hydroxyprogesterone (VI). It is felt however that with the acetylation necessary for the separation of these two compounds from one another, a more rigorous purification of testosterone (XVI), and 17α-hydroxyprogesterone (VI) has been achieved. Further work on these four neutral steroids was not feasible because of the low levels of radioactivity in the eluates from the final paper chromatograms.

Some idea of the effect of the presence of human menopausal gonadotrophin in the incubation medium may be reached by a comparison of the minimum incorporation of [1-^{14}C] acetate into each steroid in Experiments IIa (with gonadotrophin) and IIb (without gonadotrophin). On the other hand no comparison should be made of the levels of activities of oestrone (XIX) and oestradiol-17β (XX) because of technical differences. Whereas in Experiment IIa the Phenols-Acids fraction (7000 cpm) was separated into an

'oestriol' fraction (970 cpm) and an 'oestrone-oestradiol' fraction (7770 cpm), giving a 125% recovery of radioactivity, in Experiment IIb on the other hand this same separation was carried out with a recovery of only 36%, the Phenols-Acids (8900 cpm) giving rise to an 'oestriol' fraction (1350 cpm) and an 'oestrone-oestradiol' fraction (1850 cpm). It is obvious that this difference in efficiency of recovery precludes the possibility of comparing the oestrones and oestradiols from Experiments IIaand IIb.

The effect of human menopausal gonadotrophin on the incorporation of ^{14}C-acetate into the four neutral steroids is shown in Table III.12. Human menopausal gonadotrophin has increased the incorporation into progesterone (II), testosterone (XVI) and 17α-hydroxyprogesterone (VI). This is in contrast to the incorporation into androstenedione (XIV) which is greater in the experiment without gonadotrophin. These effects may have been caused however, not by the presence or absence of human menopausal gonadotrophin but rather by a metabolic difference in the tissue. The tissue in Experiment IIa came from the left ovary whereas tissue from the right ovary was used in Experiment IIb. There is a possibility of the presence of a quantitative difference in the enzyme activity between the two ovaries, particularly if some error in metabolism is present.

Table III. 12. Effect of human menopausal gonadotrophin (HMG) on the incorporation of [1-^{14}C] acetate into androstenedione, progesterone, testosterone and 17α-hydroxyprogesterone

Steroid	Minimum Incorporation of [1-^{14}C] acetate	
	Experiment IIa (with HMG)	Experiment IIb (without HMG)
Androstenedione	0.0004%	0.0010%
Progesterone	0.0011%	0.0003%
Testosterone	0.0020%	0.0010%
17α-Hydroxy-progesterone	0.0021%	0.0008%

Since the preliminary report of this present study appeared (O'Donnell and McCaig, 1959) several authors have published the results of *in vitro* incubations of ovaries including those from women with the Stein-Levanthal syndrome. Wotiz and Lemon (1958) reported that slices of several human ovaries including Stein-Levanthal ovaries converted [1-^{14}C] acetate to ^{14}C-oestrone (XIX) and ^{14}C-oestradiol-17β (XX). However, it is not clear from this abstract whether the Stein-Levanthal ovaries were included in the group of ovaries capable of synthesising oestrone (XIX) and oestradiol (XX). Slices of Stein-Levanthal ovaries have been shown by Goldzieher and Axelrod (1960) to convert ^{14}C-progesterone (II) to oestrone (XIX), oestradiol-17β (XX), 17α-hydroxyprogesterone (VI), testosterone (XVI) and androstenedione (XIV). These results support those presented in this thesis. The finding of testosterone (XVI) in both these studies is in contrast to the data of Sweat *et al.*, (1960), in which no evidence could be found for the synthesis of testosterone (XVI) in human ovarian tissue. Sweat and his co-workers, however, do report the synthesis of oestrone (XIX), oestradiol-17β (XX), androstenedione (XIV) and 17α-hydroxyprogesterone (VI) from ^{14}C-progesterone (II). In addition Sweat *et al.* (1960) present evidence for the synthesis of ^{14}C-progesterone from [1-^{14}C] acetate in bovine ovarian tissue.

The results of Lanthier and Sandor (1960) may point to one possible explanation of the excessive androgen production causing the hirsutism associated with the Stein-Levanthal syndrome. After incubation of slices of normal ovaries Lanthier and Sandor detected trace amounts of androstenedione (XIV) in the medium, especially when the tissue was incubated in the presence of a precursor (pregnenolone (XI) or 17α-hydroxyprogesterone (VI)) and human chorionic gonadotrophin. Slices of Stein-Levanthal ovaries incubated under the same conditions gave rise to increased amounts of androstenedione (XIV) in the medium as compared with the normal ovaries.

However excessive androgen production by the ovary does not appear to explain all the features of this syndrome. The elevated urinary 17-ketosteroids found by several workers (Perloff et al., 1957; Gallagher et al., 1958; Lanthier, 1960) and the suppression of this level by corticosteroids remind us once more of the possibility of a precursor elaborated from the adrenal cortex.

Section IV : Incubation of feminising adrenal tumour with sodium [1-^{14}C] acetate and [4-^{14}C] progesterone.

1. Introduction

The literature concerning the rare condition of adrenal carcinoma with gynecomastia and feminisation has been reviewed by Wallach et al., (1957). Subsequent to the thirty-four cases reviewed by these authors, several other feminising adrenal tumours have been reported (Wolf et al., 1958; Snaith, 1958; Keller et al., 1958; Tourneur et al., 1960; Ferrier et al., 1960). Because of the rarity and the individual clinical manifestations of this condition, the symptoms which cause the patient to present himself are very varied, although gynecomastia is usually present, often preceding the appearance of other symptoms by many months. However, in almost every case in which the urinary oestrogens have been determined, either by a chemical method or by a bioassay, they have been found to be higher than normal. Removal of the tumour results in a dramatic fall in the level of urinary oestrogens. Pregnane-3α:20α-diol (pregnanediol, XXVII) has been found in elevated amounts in the urine of some patients before operation. In some, but not all cases large amounts of urinary

17-ketosteroids have been found. In instances where these 17-ketosteroids have been fractionated large amounts of dehydroepiandrosterone (XIII) and aetiocholanolone (XXVI) have been found. Removal of the tumour also diminished the high levels of 17-ketosteroids.

The foregoing laboratory findings suggest that these adrenal tumours synthesise steroids in large amounts, a large proportion of these being aromatised in ring A to give oestrogens. However it is possible that these oestrogens are in fact formed from the adrenal steroids in tissues other than the adrenal cortex e.g. liver tissue. Much interest was aroused when Salhanick and Berliner (1957) reported the isolation of steroids from a feminising adrenal tumour. They detected eight steroids from the 920 g. tumour, two of which were identified as progesterone (II, 850 µg.) and 3-hydroxyoestra-1:3:5(10):6:8-pentaen-17-one (equilenin, XXVIII). This was the first report of the isolation of equilenin from a human source.

Prior to the initiation of the studies to be presented in this thesis, only one series of *in vitro* incubations of slices of feminising adrenal carcinoma had been reported (Unpublished note cited in Baggett *et al.*, 1956). Subsequently the results of these incubations were published in full by Baggett *et al.*, (1959). When Baggett and his co-workers incubated

slices of the tumour with [1-^{14}C] acetate (50 μc), no measurable radioactivity was detected in the oestrogens, nor could any labelled neutral steroids be isolated. Incubation of slices with [3-^{14}C] testosterone (XVI) resulted in radioactive oestrone (XIX), oestradiol-17β (XX) and in a compound very similar to oestriol (XXI) but which could be separated from oestriol by either methylation or a 250-transfer counter current distribution. These observations show that this carcinoma was capable of synthesising oestrogens.

The possibility that the adrenal gland is capable of synthesising oestrogens has been supported by the isolation of oestrogens in extracts of adrenal tissue. Beall (1939) isolated oestrone (XIX) from bovine adrenal extracts. The isolation of equilenin (XXVIII) from a feminising adrenal tumour (Salhanick and Berliner, 1957) has been described above and Keller (1958) reports tentative evidence for the presence of oestradiol-17β (XX) and oestrone (XIX) in extracts from an adrenal carcinoma. Oestrogen biosynthesis in the adrenal is also suggested by the finding of urinary oestrogens persisting after bilateral ovariectomy. However these oestrogens do disappear on subsequent bilateral adrenalectomy.

Synthesis of oestrogens in adrenal tissue was demonstrated by Meyer (1955) when he isolated oestrone (XIX) after incubation of bovine adrenals with

19-hydroxyandrostenedione (XXIV). Thus it appears that the adrenal, like the ovary and placenta, is capable of synthesising oestrogens.

The synthesis of corticosteroids, progesterone and androgens in adrenal tissue has been discussed in Section I.

In the following studies, tissue was obtained from a 39-year-old man undergoing surgery for the removal of a feminising adrenal tumour (wet wt. 1100 g.). Prior to the operation, the urinary levels of oestrogens, pregnanediol (XXVII) and 17-ketosteroids were elevated, but diminished markedly on removal of the tumour. Before operation, the intravenous infusion of ACTH caused only a slight elevation in the already high level of plasma 17-hydroxycorticosteroids. Following extirpation of the tumour, the remaining adrenal showed a response to exogenous ACTH although to a lesser extent than in normal individuals. A comprehensive study was made on an extract of a portion (501 g.) of tumour but no evidence was obtained for the presence of any steroids (O'Donnell et al., 1960).

Slices and homogenate preparations of the tumour were incubated with [1-^{14}C] acetate and [4-^{14}C] progesterone. In the following report, the investigations on the capacity of the tissue to synthesise steroids are presented.

2a. Incubation conditions.

Tumour slices (3-4 g.) were incubated in 25 ml. of Krebs-Ringer phosphate buffer, pH 7.4, containing glucose (0.01 M), fumarate (0.01 M) and nicotinamide (0.04 M) together with the substrates and cofactors listed in Table IV.1. Incubation was carried out for 3 hours with shaking, in an atmosphere of oxygen at 37° C.

50.6 g. of tumour tissue was homogenised in Krebs-Ringer phosphate buffer (pH 7.4). The total volume of the homogenate was 150 ml. (340 mg. wet weight tissue per ml.; 53.9 mg. dry weight tissue per ml.).

25 ml. portions of the homogenate were incubated for 3 hours with shaking, under an atmosphere of oxygen, together with glucose (0.01 M), nicotinamide (0.04 M), fumarate (0.01 M), adenosine triphosphate (0.005 M) and triphosphopyridine nucleotide (0.0005 M). The following substrates were added:

1. sodium [1-^{14}C] acetate (25 mg.; 50 μc)
2. [4-^{14}C] progesterone (30.8 μg.; 2 μc), dissolved in propylene glycol (0.2 ml.).

2b. Extraction and Purification Procedure.

In the experiments using tissue slices, the slices were homogenised in water (20 ml. approx.) and

Table IV. 1. Incubation of feminising adrenal tumour slices with various substrates and cofactors.

Expt. No.	Wt. slices (g.)	Substrate	Cofactor
1	3.10	sodium [1-^{14}C] acetate 25 mg.; 50 µc	-
2	3.59	sodium [1-^{14}C] acetate 25 mg.; 50 µc	ACTH 30 i.u.
3	3.69	sodium [1-^{14}C] acetate 25 mg.; 50 µc	Human menopausal gonadotrophin (HMG) 25 mg.; 25 units
4	3.56	[4-^{14}C] progesterone* 30.8 µg.; 2 µc	-

* The [4-^{14}C] progesterone was dissolved in propylene glycol (0.2 ml.).

this homogenate added to the incubation medium. The homogenates from all experiments were extracted with ethyl acetate (3 x 50 ml.) and chloroform (3 x 50 ml.). The residue was partitioned between heptane (100 ml.) and 90% methanol (100 ml.). The phases were separated and each was shaken with 50 ml. of fresh opposite phase. These two 50 ml. phases were shaken together. The combined heptane phases and the combined 90% methanol phases were evaporated to dryness in vacuo.

The methanol fraction of the ^{14}C-acetate (homogenate) experiment was resolved by paper chromatography using the toluene-propylene glycol and ligroin-propylene glycol solvent systems. No steroids could be detected. Thus it was found necessary to add unlabelled steroids as carriers.

Addition of carrier steroids.

The following carriers (1 mg. approx.) were added to the ^{14}C-progesterone (homogenate), ^{14}C-acetate (control), ^{14}C-acetate (HMG) and ^{14}C-progesterone (slice) experiments: cortisol (VIII), corticosterone (IV), 11-deoxycortisol (VII), 17α-hydroxyprogesterone (VI), deoxycorticosterone (III), dehydroepiandrosterone (XIII), androstenedione (XIV), oestradiol (XX), oestrone (XIX) and oestriol (XXI). These carriers were added to the crude ethyl acetate-chloroform extract. The

same carriers with the exception of oestriol were added to the methanol fraction of the ^{14}C-acetate (ACTH) incubation.

Column Chromatography.

The steroids were partially resolved by column chromatography on silica gel ("Davison" 923, 100/200 mesh).

To a column, 1 cm. diameter, of silica gel (8-10 g.) in benzene, the residue from the 90% methanol fraction was added dissolved in benzene (25 ml.). The following elutions were made: benzene (10-25 ml.; bright yellow); ethyl acetate in benzene (v/v):1% (50-70 ml.), 2% (70-85 ml.), 4% (70-100 ml.), 10% (100-120 ml.), 20% (80-100 ml.) and 40% (100 ml.); ethyl acetate (100-120 ml.); ethanol (75-100 ml.; straw coloured) and chloroform (100 ml.). Except where noted otherwise, the eluates obtained were colourless. From the absorption spectra in methanol of the residues from these eluates, it was found that the steroids appeared in the eluates 10% (v/v) ethyl acetate in benzene to 100% ethyl acetate. The ethanol eluate (straw coloured) was shown to consist almost entirely of nicotinamide.

Paper Chromatography.

The steroids of the 10%, 20% and 40% (v/v) ethyl acetate in benzene and the 100% ethyl acetate eluates were resolved by paper chromatography using the toluene-propylene glycol and ligroin-propylene glycol solvent systems.

They were first run in the solvent system toluene-propylene glycol for 48 hours, thus separating oestriol (XXI), cortisol (VIII), oestradiol (XX), 11-deoxycortisol (VII) and corticosterone (IV). By paper chromatography of the overflow in ligroin-propylene glycol for 48 hours, oestrone (XIX), 17α-hydroxyprogesterone (VI) and deoxycorticosterone (III) were separated. The overflow from this was in turn chromatographed in ligroin-propylene glycol until the solvent front reached the end of the papergram (about 8 hours). On this chromatogram, androstenedione and progesterone were separated. A diagram of the separation of the steroids by paper chromatographic systems is shown in Fig. IV.1.

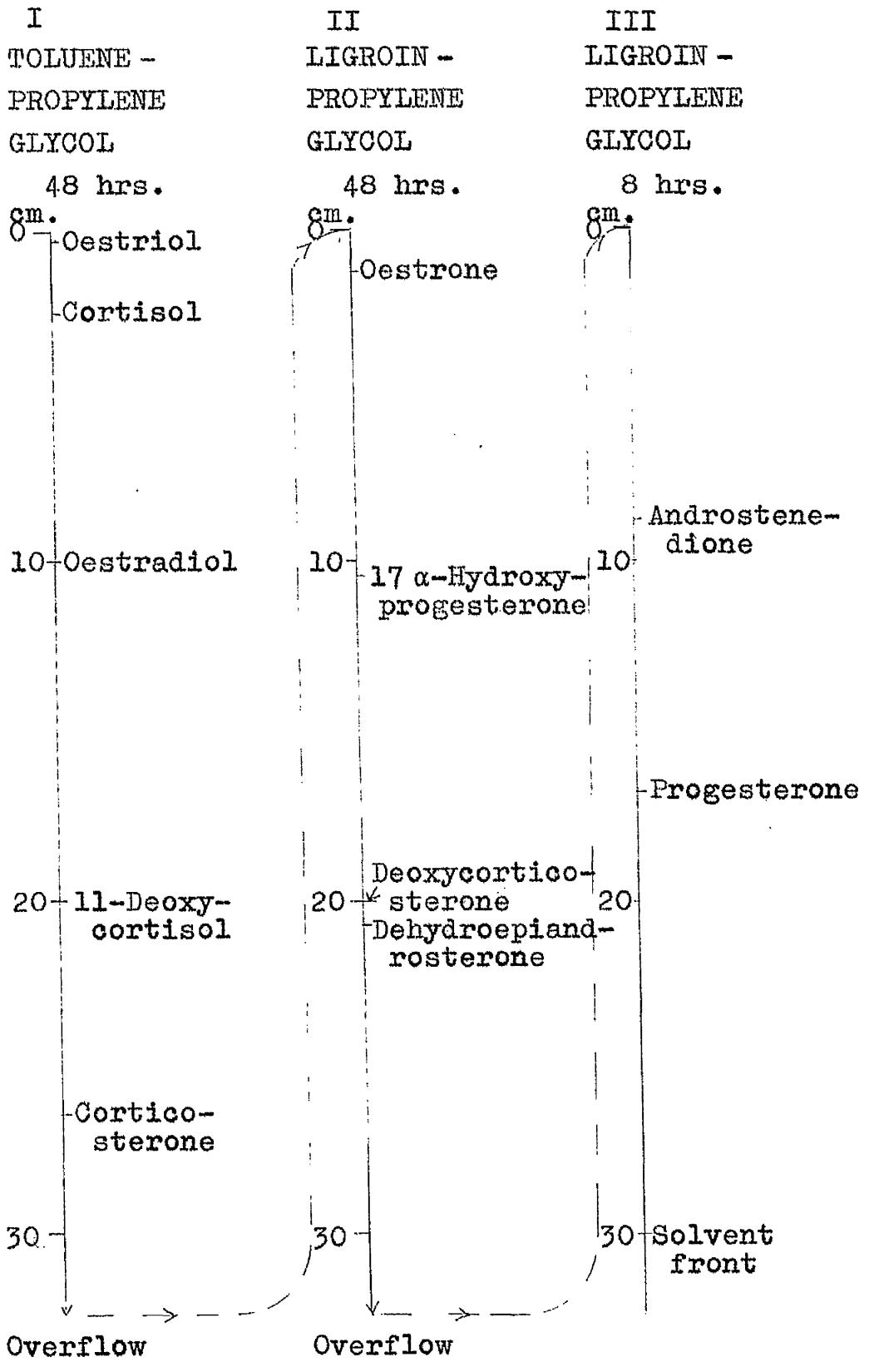


Fig. IV.1. Separation of steroids by paper chromatography.

3. Results

(i) Incubation of slices with [1-^{14}C] acetate in the presence of ACTH.

The detected steroids were further purified by repeated paper chromatography. When the specific activities of androstenedione (XIV), 17α-hydroxyprogesterone (VI) and progesterone (II) were constant, each was dissolved in pyridine and acetic anhydride was added. Subsequent chromatograms separated any easily acetylable impurity from the steroid being studied.

a) Cortisol (VIII).

After three successive paper chromatograms in the toluene-propylene glycol solvent system, the specific activity of the cortisol was constant at 1800 cpm per mg. The specific activities of the material isolated from each chromatogram are shown in Table IV.2. The spectrum of a sulphuric acid chromogen of the final material (37 μg./3 ml., H_2SO_4) was identical with that of authentic cortisol (Section II):

Max. mμ (O.D.): 234 (0.540) [I]; 279 (0.500); 392 (0.240); 466 (0.305).

Min. mμ (O.D.): 251 (0.369); 348 (0.144); 410 (0.201).

Table IV. 2 Specific activities of cortisol from successive paper chromatograms. Incubation of tumour slices with [1-^{14}C] acetate and ACTH.

Chromatogram	Sp.act.(cpm per mg.)
1	2453
2	2060
3	1800
Carrier added	1058 µg.
Minimum incorporation of [1-^{14}C] acetate	0.0029%

b) Androstenedione (XIV).

After four paper chromatograms in the solvent system ligroin-propylene glycol the specific activity of androstendione was constant at 931 cpm per mg. Acetylation and subsequent chromatography freed the material of a radioactive impurity. After a further three paper chromatograms in the same system the specific activity of the androstenedione was constant at 203 cpm per mg. The specific activities of the material after each chromatogram are shown in Table IV.3. The spectrum of a sulphuric acid chromogen of the material (24.5 µg./3 ml. H_2SO_4) before acetylation was identical with that of authentic androstenedione (Section II):

Max. mµ (O.D.): 295 (0.628)

Min. mµ (O.D.): 236 (0.129)

c) 17α-Hydroxyprogesterone (VI).

Four paper chromatograms, the first two in the ligroin-propylene glycol, the second two in the toluene-propylene glycol solvent system yielded material of specific activity 8385 cpm per mg. Acetylation and another chromatogram in the solvent system toluene-propylene glycol caused the specific activity of the 17α-hydroxyprogesterone to drop to 1471 cpm per mg. The specific activities of the material obtained from

Table IV. 3. Specific activities of androstenedione from successive paper chromatograms. Incubation of tumour slices with [1-^{14}C] acetate and ACTH.

Chromatogram	Sp.act.(cpm per mg.)
1	2127
2	1218
3	1014
4	931
Acetylation + 5	335
6	175
7	203
Carrier added	1053 µg.
Minimum incorporation of [1-^{14}C] acetate	0.0003%

each chromatogram are shown in Table IV.4. Insufficient material remained for further purification. The spectrum of a sulphuric acid chromogen of material (24.8 µg./3 ml. H_2SO_4) before acetylation was identical with that of authentic 17α-hydroxyprogesterone (Section II):
Max. mµ (O.D.): 244 (0.232) [I]; 288 (0.509); 428 (0.263).
Min. mµ (O.D.): 233 (0.223); 345 (0.069).

d) Progesterone (II)

Progesterone, after four chromatograms in the ligroin-propylene glycol solvent system had a constant specific activity of 1727 cpm per mg. Acetylation and a further three chromatograms in the same system yielded progesterone of constant specific activity 694 cpm per mg. The specific activities of the material obtained from each chromatogram are shown in Table IV.5. The spectrum of a sulphuric acid chromogen of material (52 µg./3 ml. H_2SO_4) before acetylation was identical with that of authentic progesterone (Section II):
Max. mµ (O.D.): 233 (0.440); 291 (1.110).
Min. mµ (O.D.): 224 (0.432); 236 (0.432).

e) Oestrone (XIX)

One paper chromatogram in the ligroin-propylene glycol and two in the toluene-propylene

Table IV. 4. Specific activities of 17α-hydroxy-progesterone from successive paper chromatograms.
Incubation of tumour slices with [1-^{14}C] acetate and ACTH.

Chromatogram	Sp.act.(cpm per mg.)
1	4482
2	17090
3	7550
4	8385
Acetylation + 5	1471
Carrier added	1008 µg.

Table IV. 5. Specific activities of progesterone from successive paper chromatograms
Incubation of tumour slices with [1-^{14}C] acetate and ACTH.

Chromatogram	Sp.act.(cpm per mg.)
1	6430
2	3286
3	1758
4	1727
Acetylation + 5	1275
6	697
7	694
Carrier added	1022 µg.
Minimum incorporation of [1-^{14}C] acetate	0.0011%

glycol solvent systems yielded oestrone of specific activity 114 cpm per mg. The specific activity of the oestrone diminished with each chromatogram step. The specific activities of the material obtained from each chromatogram are shown in Table IV.6. The spectrum of a sulphuric acid chromogen of the final material (47.2 µg./3 ml. H_2SO_4) was identical with that of authentic oestrone (Section II):

Max. mµ (O.D.): 227 (0.497)[I]; 300 (0.671); 446 (0.390).

Min. mµ (O.D.): 256 (0.157); 375 (0.059).

f) Oestradiol-17β (XX).

Two paper chromatograms in the toluene-propylene glycol solvent system yielded material of specific activity 46.5 cpm per mg. There was no constancy in the specific activities. The results from both chromatograms are shown in Table IV.7. The spectrum of a sulphuric acid chromogen of the final material (44.7 µg./3 ml. H_2SO_4) was identical with that of authentic oestradiol (Section II):

Max. mµ (O.D.): 275 (0.269)[I]; 282 (0.274); 302 (0.300); 366 (0.772); 422 (0.669).

Min. mµ (O.D.): 258 (0.224); 287 (0.270); 319 (0.254); 389 (0.407).

Table IV. 6 Specific activities of oestrone from successive paper chromatograms. Incubation of tumour slices with [1-^{14}C] acetate and ACTH.

Chromatogram	Sp.act.(cpm per mg.)
1	2074
2	420
3	114
Carrier added	970 µg.

Table IV. 7. Specific activities of oestradiol-17β from successive paper chromatograms. Incubation of tumour slices with [1-^{14}C] acetate and ACTH.

Chromatogram	Sp.act.(cpm per mg.)
1	122
2	46.5
Carrier added	1004 µg.

(ii) Incubation of Slices with [1-^{14}C] acetate.

a) Cortisol (VIII)

Of the 997 µg. carrier cortisol originally added, 558 µg. was recovered of specific activity 5116 cpm per mg. Additional carrier cortisol was purified by column chromatography on silica gel, followed by repeated crystallisations (final m.p. 189-198.5°). On a paper chromatogram, it behaved as a homogenous substance. 15.19 mg. of this material was added to the radioactive material to give a total weight of 16.12 mg. of specific activity 123 cpm per mg. Two crystallisations were performed, the first from aqueous methanol, the second from ether-methanol. The final crop of crystals (8.31 mg.) had a specific activity of less than 5 cpm per mg. The results are shown in Table IV.8. The spectrum of a solution of the final material in methanol showed a maximum at 242 mµ (ε = 16,550). The spectrum of a sulphuric acid chromogen of the final material (33.24 µg./3 ml. H_2SO_4) was identical with that of authentic cortisol (Section II):

Max. mµ (O.D.): 229 (0.570)[I]; 270 (0.502);
394 (0.226); 471 (0.330).

Min. mµ (O.D.): 281 (0.368); 337 (0.117); 408 (0.201).

Table IV. 8. Results of crystallisation of cortisol: weights, m.p.'s and specific activities of crystals; weights and specific activities of mother liquors. Incubation of tumour slices with [1-^{14}C] acetate.

	C R Y S T A L S			MOTHER LIQUORS	
Cryst.	Wt. (mg.)	M.p.	Sp.act. (cpm per mg.)	Wt. (mg.)	Sp.act. (cpm per mg.)
1	11.69	190-197°	32.9	3.69	408
2	8.31	186-194°	5	2.60	94.5

b) Androstenedione (XIV).

After elution from the initial chromatogram in the solvent system ligroin-propylene glycol, the androstenedione was dissolved in pyridine and acetic anhydride added. The androstenedione was further purified by two paper chromatograms in the ligroin-propylene glycol solvent system and had a final specific activity of approximately 20 cpm per mg. The specific activity of the androstenedione diminished at each stage of the purification procedure. The specific activities of the material obtained from each of these chromatograms are shown in Table IV.9. The spectrum of a sulphuric acid chromogen of the final material (12 µg./3 ml. H_2SO_4) was identical with that of authentic androstenedione (Section II):

Max. mµ (O.D.): 292 (0.269).

Min. mµ (O.D.): 236 (0.061).

c) 17α-Hydroxyprogesterone (VI).

The specific activity of the 17α-hydroxy-progesterone recovered from the initial chromatogram in the ligroin-propylene glycol solvent system was 983 cpm per mg. After subjection to acetylation conditions and a further chromatogram in the ligroin-propylene glycol solvent system, the specific activity of the 17α-hydroxyprogesterone decreased to 287 cpm per mg.

Table IV. 9. Specific activities of androstenedione from successive paper chromatograms. Incubation of tumour slices with [1-^{14}C] acetate.

Chromatogram	Sp.act.(cpm per mg.)
1	1215
Acetylation + 2	66.3
3	20 (approx.)
Carrier added	987 µg.

Unfortunately the amount of material remaining did not permit further purification.

d) Progesterone (II).

After the initial paper chromatogram in the ligroin-propylene glycol solvent system, the progesterone was subjected to acetylation conditions and purified by two further chromatograms in ligroin-propylene glycol. The progesterone then exhibited a constant specific activity of 112 cpm per mg. The specific activities of the material obtained from these chromatograms are presented in Table IV.10. The spectrum of a sulphuric acid chromogen of the final material (25.6 µg./3 ml. H_2SO_4) was identical with that of authentic progesterone (Section II):

Max. mµ (O.D.): 291 (0.410).

Min. mµ (O.D.): 224 (0.159).

e) Oestrone (XIX).

Oestrone was chromatographed twice in the ligroin-propylene glycol solvent system, then acetylated and purified by a paper chromatogram in ligroin-propylene glycol, yielding material of final specific activity 154 cpm per mg. free oestrone. At each stage, the specific activity of the oestrone diminished. The specific activities of the material obtained from

Table IV. 10. Specific activities of progesterone from successive paper chromatograms. Incubation of tumour slices with [1-^{14}C] acetate.

Chromatogram	Sp.act.(cpm per mg.)
1	1144
Acetylation + 2	105
3	112
Carrier added	1056 µg.
Minimum incorporation of [1-^{14}C] acetate	0.00018%

these chromatograms are shown in Table IV.11. The spectrum of a sulphuric acid chromogen of the final material (31.4 µg. acetate/3 ml. H_2SO_4) was identical with that of authentic oestrone acetate (Section II):
Max. mµ (O.D.): 228 (0.260) [I]; 301 (0.342); 448 (0.231).
Min. mµ (O.D.): 250 (0.094); 378 (0.041).

f) Oestradiol-17β (XX).

Two paper chromatograms of oestradiol were run in the toluene-propylene glycol solvent system yielding oestradiol of final specific activity 92.2 cpm per mg. No constancy of specific activity was achieved. The specific activities of the material obtained from these chromatograms are shown in Table IV.12. The spectrum of a sulphuric acid chromogen of the final material (25.4 µg./3 ml. H_2SO_4) was identical with that of authentic oestradiol (Section II):
Max. mµ (O.D.): 234 (0.247) [I]; 269 (0.167);
302 (0.161); 366 (0.410); 426 (0.518);
446 (0.330) [I].
Min. mµ (O.D.): 252 (0.155); 285 (0.150); 320 (0.131);
390 (0.275).

g) Oestriol (XXI).

After recovery from the initial chromatogram in the toluene-propylene glycol solvent system,

Table IV. 11. Specific activities of oestrone from successive paper chromatograms. Incubation of tumour slices with [1-^{14}C] acetate.

Chromatogram	Sp.act.(cpm per mg.)
1	1289
2	1418
Acetylation + 3	154 (calculated as free oestrone)
Carrier added	1111 µg.

Table IV. 12. Specific activities of oestradiol-17β from successive paper chromatograms. Incubation of tumour slices with [1-^{14}C] acetate.

Chromatogram	Sp.act.(cpm per mg.)
1	265
2	92.2
Carrier added	1084 µg.

oestriol was acetylated and purified by a paper chromatogram in the ligroin-propylene glycol system. The specific activity of the final material was 103 cpm per mg. free oestriol. The specific activity of the oestriol was not constant. The specific activities of the material obtained from these chromatograms are shown in Table IV.13. The spectrum of a sulphuric acid chromogen of the final material (27.2 µg. acetate/ ml. H_2SO_4) was identical with that of authentic oestriol acetate (Section II):

Max. mµ (O.D.): 231 (0.500) [I]; 305 (0.332); 354 (0.076); 448 (0.107).

Min. mµ (O.D.): 262 (0.0825); 333 (0.070); 387 (0.050).

Table IV. 13. Specific activities of oestriol after successive paper chromatograms. Incubation of tumour slices with [1-^{14}C] acetate.

Chromatogram	Sp.act.(cpm per mg.)
1	5962
Acetylation + 2	103 (calculated as free oestriol)
Carrier added	1199 µg.

(iii) Incubation of slices with [1-^{14}C] acetate in the presence of human menopausal gonadotrophin.

a) Cortisol (VIII).

After recovery from the initial paper chromatogram in toluene-propylene glycol, cortisol was acetylated and purified by two further chromatograms in the same solvent system. Constancy in the specific activity was not achieved. The final material had a specific activity of 135 cpm per mg. free cortisol. The specific activities of the material obtained from each chromatogram are shown in Table IV.14. The spectrum of a sulphuric acid chromogen of the final material (10.20 µg. acetate/ml. H_2SO_4) was identical with that of authentic cortisol 21-monoacetate (Section II):

Max. mµ (O.D.): 234 (0.350); 241 (0.330); 252 (0.231) [I]; 285 (0.567); 389 (0.208); 428 (0.146).

Min. mµ (O.D.): 230 (0.341); 239 (0.326); 250 (0.221); 360 (0.179).

b) Androstenedione (XIV).

After recovery from the initial paper chromatogram in the ligroin-propylene glycol solvent system, androstenedione was dissolved in pyridine and acetic anhydride added. Three subsequent chromatograms

Table IV. 14. Specific activities of cortisol from successive paper chromatograms. Incubation of tumour slices with [1-^{14}C] acetate and HMG.

Chromatogram	Sp.act.(cpm per mg.)
1	850
Acetylation + 2	1005 (calculated as free cortisol)
3	135 (calculated as free cortisol)
Carrier added	997 µg.

in the ligroin-propylene glycol solvent system were performed. The specific activity of the androstenedione fell at each stage. The specific activity of the final material was 19.7 cpm per mg. The specific activities of the material obtained from each chromatogram are shown in Table IV.15. The spectrum of a sulphuric acid chromogen of the final material (23.4 µg./3 ml. H_2SO_4) was identical with that of authentic androstenedione (Section II):

Max. mµ (O.D.): 293 (0.472).

Min. mµ (O.D.): 235 (0.095).

c) 17α-Hydroxyprogesterone (VI).

After recovery from the initial paper chromatogram, 17α-hydroxyprogesterone was subjected to acetylation conditions. Two subsequent paper chromatograms were performed in the ligroin-propylene glycol solvent system. The specific activity of the final material was constant at 427 cpm per mg. The specific activities of the material obtained from each chromatogram are shown in Table IV.16. The spectrum of a sulphuric acid chromogen of the final material (12 µg./3 ml. H_2SO_4) was identical with that of authentic material (Section II):

Max. mµ (O.D.): 287 (0.260); 430 (0.108).

Min. mµ (O.D.): 241 (0.092); 347 (0.043).

Table IV. 15. Specific activities of androstenedione from successive paper chromatograms. Incubation of slices with [1-^{14}C] acetate and HMG.

Chromatogram	Sp.act.(cpm per mg.)
1	1859
Acetylation + 2	98.5
3	39.5
4	19.7
Carrier added	987 µg.

Table IV. 16. Specific activities of 17α-hydroxy-progesterone from successive paper chromatograms.
Incubation of slices with [1-^{14}C] acetate and HMG.

Chromatogram	Sp.act.(cpm per mg.)
1	1265
Acetylation + 2	426
3	427
Carrier added	884 µg.
Minimum incorporation of [1-^{14}C] acetate	0.0057%

d) Progesterone (II).

After recovery from the initial paper chromatogram, progesterone was subjected to acetylation conditions and subsequently chromatographed three times in the solvent system ligroin-propylene glycol. The specific activity of the final material was constant at 177 cpm per mg. The specific activities are shown in Table IV.17. The spectrum of a sulphuric acid chromogen of the final material (24.8 µg./3 ml.) was identical with that of authentic progesterone.
(Section II):
Max. mµ (O.D.): 292 (0.351).
Min. mµ (O.D.): None.

e) Oestrone (XIX).

After two paper chromatograms, first in ligroin-propylene glycol and then in toluene-propylene glycol, the oestrone was acetylated. The reaction product was chromatographed twice in the solvent system ligroin-propylene glycol. The specific activity of the final material was 548 cpm per mg. free oestrone. Constancy in the specific activity was not achieved. The specific activities of the material obtained from each chromatogram are shown in Table IV.18. The spectrum of a sulphuric acid chromogen of the final material (30.2 µg. acetate/3 ml. H_2SO_4) was identical

Table IV. 17. Specific activities of progesterone from successive paper chromatograms. Incubation of slices with [1-^{14}C] acetate and HMG.

Chromatogram	Sp.act.(cpm per mg.)
1	2195
Acetylation + 2	277
3	164
4	177
Carrier added	1056 µg.
Minimum incorporation of [1-^{14}C] acetate	0.00028%

Table IV. 18. Specific activities of oestrone from successive paper chromatograms. Incubation of slices with [1-^{14}C] acetate and HMG.

Chromatogram	Sp.act.(cpm per mg.)
1	2993
2	5266
Acetylation + 3	861 (calculated as free oestrone)
4	548 (calculated as free oestrone)
Carrier added	1111 µg.

with that of authentic oestrone acetate (Section II):

Max. mμ (O.D.): 230 (0.192) [I]; 300 (0.276);
450 (0.186).

Min. mμ (O.D.): 250 (0.074); 365 (0.060).

f) Oestradiol-17β (XX).

After two paper chromatograms in the solvent system toluene-propylene glycol, the specific activity of the oestradiol was 55.5 cpm per mg. Constancy in the specific activity was not achieved. The specific activities of the material obtained from each chromatogram are shown in Table IV.19. The spectrum of a sulphuric acid chromogen of the final material (32.8 μg./3 ml. H_2SO_4) was identical with that of authentic oestradiol (Section II):

Max. mμ (O.D.): 256 (0.403); 301 (0.189); 366 (0.441);
426 (0.562); 446 (0.345) [I].

Min. mμ (O.D.): 237 (0.290); 284 (0.176); 320 (0.151);
390 (0.291).

g) Oestriol (XXI).

After recovery from the initial paper chromatogram, oestriol was acetylated and subsequently chromatographed three times. The specific activity of the final material was 202 cpm per mg. free oestriol. The specific activity diminished at each

Table IV. 19. Specific activities of oestradiol from successive paper chromatograms. Incubation of slices with [1-^{14}C] acetate and HMG.

Chromatogram	Sp.act.(cpm per mg.)
1	93.4
2	55.5
Carrier added	1084 µg.

stage of the purification. The specific activities of the material obtained from each chromatogram are shown in Table IV.20. The spectrum of a sulphuric acid chromogen of the final material (20.0 µg. acetate/ml. H_2SO_4) was identical with that of authentic oestriol triacetate (Section II):

Max. mµ (O.D.): 231 (0.240) [I]; 304 (0.169); 354 (0.0925); 449 (0.070).

Min. mµ (O.D.): 266 (0.095); 340 (0.090); 420 (0.060).

Table IV. 20. Specific activities of oestriol from successive paper chromatograms. Incubation of slices with [1-^{14}C] acetate and HMG.

Chromatogram	Sp.act.(cpm per mg.)
1	786
Acetylation + 2	470 (calculated as free oestriol)
3	266 (calculated as free oestriol)
4	202 (calculated as free oestriol)
Carrier added	1199 µg.

(iv) Incubation of slices with [4-^{14}C] progesterone.

In this experiment, additional carrier material was added to each steroid, and the steroid purified by repeated recrystallisation. In the cases where constant specific activity was achieved, a derivative of the steroid was prepared and crystallised.

a) Cortisol (VIII).

Of the 997 µg. cortisol carrier originally added, 661.5 µg. of specific activity 148,000 cpm per mg. was recovered. Additional carrier cortisol (29.41 mg., m.p. 189-198.5°, purified as described in Section IV, p. 50) was added to the radioactive material to give a total weight of 30.91 mg., specific activity 3292 cpm per mg. Four crystallisations were performed and the results are shown in Table IV.21. The specific activity of the final crop of crystals was 1174 cpm per mg. The cortisol was acetylated and the product crystallised twice. Under the conditions used, only the hydroxyl group at C-21 of cortisol is acetylated. The specific activities of the crystals and the mother liquors are shown in Table IV.21. The specific activity of the final material was 1162 cpm calculated per mg. free cortisol. The m.p. of a mixture of the final crystals with authentic cortisol 21-monoacetate (m.p. 200-212°) was 199-212°. A solution of the final

Table IV. 21. Specific activities, weights and mp.'s of cortisol after crystallisation, acetylation and subsequent crystallisations.
Incubation of slices with [4-^{14}C] progesterone.

Carrier added	997 µg.
Cortisol recovered	661.5 µg.; 148000 cpm per mg.
Additional carrier added	29.41 mg.
Total material	30.91 mg.; 3292 cpm per mg.

		CRYSTALS			MOTHER LIQUOR	
Cryst.	Solvent	Wt. (mg.)	M.p.	Sp.act*	Wt. (mg.)	Sp.act*
1	Aqueous methanol	26.52	182-193°	2170	4.29	10586
2	Ether-methanol	21.93	184-188.5°	1752	3.85	5327
3	Ligroin-ethanol	15.97	185-192°	1250	6.00	3052
4	Ligroin-ethanol	10.61	183.5-197°	1174	5.01	1443
Acetylation Product		11.92	-	1029		
1	Aqueous methanol	9.25	202-210°	1128	2.01	1305
2	Aqueous methanol	7.31	201-210°	1162	1.32	1276

*Cpm per mg. free cortisol

material in methanol exhibited λ_{max} 241-242 mμ (ε_{max} = 17,140).

b) Androstenedione (XIV).

Carrier androstenedione was purified by repeated crystallisations to give material of m.p. 169.5-171°.

Of the 1026 μg. carrier androstenedione originally added, 779 μg. was recovered, of specific activity 11,160 cpm per mg. Additional carrier material (19.22 mg.) was added to the radioactive material to give a total weight of 20.30 mg., specific activity of 539 cpm per mg. calculated. The material was crystallised three times and the specific activity of the final material was 353 cpm per mg. The bis-dinitrophenylhydrazone of androstenedione was prepared (Section II), purified by silica gel column chromatography and crystallised. The final specific activity of the derivative was 369 cpm per mg. free androstenedione. The crystals melted with decomposition at about 300°. Authentic androstenedione bis-dinitrophenylhydrazone, and a mixture of the two, also melted with decomposition at about 300°. The spectrum of a solution of the crystals in chloroform was identical with that of authentic material:

Max. mμ (ε): 259 (ε=30,070); 290 (ε=14,185) [I]; 378-379 (ε=49,270).

Min. mμ (ε): 312-313 (ε=7,801).

The androstenedione bis-dinitrophenylhydrazone was hydrolysed (Section II) and the androstenedione purified by two paper chromatograms in the solvent system ligroin-propylene glycol. The specific activity was 403 cpm per mg. The spectrum of a sulphuric acid chromogen of the final material (23.9 μg./3 ml. H_2SO_4) was identical with that of authentic androstenedione (Section II):

Max. mμ (O.D.): 292 (0.540)

Min. mμ (O.D.): 237 (0.102)

The specific activities of the material at every stage of the purification are shown in Table IV.22.

c) 17α-Hydroxyprogesterone (VI).

Carrier 17α-hydroxyprogesterone was purified by crystallisation from aqueous methanol to give material which exhibited a m.p. 203-207°.

Of the 750.7 μg. carrier 17α-hydroxyprogesterone originally added, 552 μg. was recovered, of specific activity 12,554 cpm per mg. Additional carrier material (25.30 mg.) was added to the radioactive material to give a total weight of 26.29 mg. of specific activity 240 cpm per mg.

Three crystallisations were performed. The specific activity after the third crystallisation was 14.40 cpm per mg. This material was subjected to the

Table IV. 22. Specific activities, weights and m.p.'s of androstenedione after crystallisation, formation of bis-dinitrophenylhydrazone (bis-DNP) and subsequent hydrolysis.
Incubation of slices with [4-^{14}C] progesterone.

Carrier added	1026 µg.
Androstenedione recovered	779 µg.; 11160 cpm per mg.
Additional carrier added	19.22 mg.
Total material	20.30 mg.; 539 cpm per mg.

		CRYSTALS			MOTHER LIQUOR	
Cryst.	Solvent	Wt. (mg.)	M.p.	Sp.act*	Wt. (mg.)	Sp.act*
1	Aqueous methanol	14.54	165-169.5°	490	5.27	1149
2	Aqueous methanol	11.66	166.5-170°	374	2.80	593
3	Aqueous methanol	8.98	167-170°	353	2.56	434
Bis-DNP Product		27.49	-	209		
Cryst.	Chloroform	9.20	300°d	369	10.60	223
Hydrolysis and Paper chromatography (2 X)		957 µg.	-	403		

* Cpm per mg. free androstenedione.

usual acetylation conditions, under which 17α-hydroxy-progesterone is not acetylated, and the resulting material after crystallisation had a specific activity of 15.03 cpm per mg. The specific activities of the crystals and mother liquors are shown in Table IV.23.

d) Progesterone (II).

Of the 966 µg. carrier progesterone added, 810 µg. was recovered of specific activity 15,358 cpm per mg. No attempt was made to establish the radiochemical homogeneity of this progesterone sample.

e) Oestrone (XIX).

Carrier oestrone was purified by crystallisation from aqueous methanol to give material of m.p. 248-251°.

Of the 1013 µg. carrier oestrone originally added, 437 µg. was recovered of specific activity 872 cpm per mg. Additional carrier oestrone (6.72 mg.) was added to the radioactive material to give 7.28 mg. total weight of specific activity 32.7 cpm per mg. One crystallisation from aqueous methanol yielded 5.24 mg. crystals (m.p. 234-242°) of negligable radioactivity. The mother liquor (1.16 mg.) had a specific activity of 204 cpm per mg. A solution of the crystals in methanol had a spectrum identical with that of

Table IV. 23. Specific activities, weights and m.p.'s of 17α-hydroxyprogesterone after successive crystallisations. Incubation of slices with [4-^{14}C] progesterone.

Carrier added	750.7 µg.
17α-Hydroxyprogesterone recovered	552 µg.; 12554 cpm per mg.
Additional carrier added	25.30 mg.
Total material	26.29 mg.; 240 cpm per mg.

		CRYSTALS			MOTHER LIQUOR	
Cryst.	Solvent	Wt. (mg.)	M.p.	Sp.act. (cpm per mg.)	Wt. (mg.)	Sp.act (cpm per mg.)
1	Aqueous methanol	20.01	206-215°	42.2	5.04	1165
2	Ether-methanol	16.74	204-212°	17.5	3.00	169
3	Chloro-form-methanol	12.77	206-212°	14.40	3.15	33.36
Acet. + 4	Aqueous methanol	9.67	200-214°	15.03	1.71	-

authentic oestrone:

λ^{MeOH}_{Max} : 281 mμ (ε = 2117)

λ^{MeOH}_{Min} : 248-249 mμ (ε = 232)

The spectrum of a sulphuric acid chromogen of the final material (52.6 μg./3 ml. H_2SO_4) was identical with that of authentic oestrone (Section II):

Max. mμ (O.D.): 229 (0.746) [I]; 299 (0.834);
448 (0.666).

Min. mμ (O.D.): 254 (0.202); 371 (0.054).

f) Oestradiol-17β (XX).

Carrier oestradiol-17β was purified by crystallisation from aqueous methanol to yield material of m.p. 175-177°.

Of the 1005 μg. oestradiol originally added as carrier, 992 μg. of specific activity 4340 cpm per mg. was recovered. Additional carrier oestradiol (15.35 mg.) was added to the radioactive material giving a total weight of 16.64 mg. of determined specific activity 254 cpm per mg. After two crystallisations the specific activity was 6.9 cpm per mg. The results are shown in Table IV.24. The mixed m.p. with authentic oestradiol (m.p. 175-177°) was 174-178°.

Table IV. 24. Specific activities, weights and m.p.'s of oestradiol after successive crystallisations.
Incubation of slices with [4-^{14}C] progesterone.

Carrier added	1005 µg.
Oestradiol recovered	992 µg.; 4340 cpm per mg.
Additional carrier added	15.35 mg.
Total material	16.64 mg.; 254 cpm per mg.

		CRYSTALS			MOTHER LIQUOR	
Cryst.	Solvent	Wt. (mg.)	M.p.	Sp.act. (cpm per mg.)	Wt. (mg.)	Sp.act. (cpm per mg.)
1	Aqueous methanol	13.04	172.5-175°	15.3	3.43	1350
2	Aqueous methanol	9.02	174-178°	6.9	-	-

The spectrum of a solution of the final material in methanol was identical with that of authentic oestradiol:

λ^{MeOH}_{Max} : 281 mμ (ε = 1983)

λ^{MeOH}_{Min} : 249 mμ (ε = 248)

The spectrum of a sulphuric acid chromogen of the final material (25.4 μg./3 ml. H_2SO_4) was identical with that of authentic oestradiol (Section II):

Max. mμ (O.D.): 231 (0.263); 281 (0.157); 302 (0.166); 366 (0.432); 424 (0.419).

Min. mμ (O.D.): 227 (0.257); 264.5 (0.123); 287 (0.155); 320 (0.145); 390 (0.240).

g) Oestriol (XXI).

Carrier oestriol was purified by crystallisations from aqueous methanol to yield material of m.p. 266-270°.

Of the 980 μg. oestriol originally added as carrier, 770 μg. was recovered of specific activity 20,644 cpm per mg. Additional carrier oestriol (15.55 mg.) was added to the radioactive material to give a total weight of 16.85 mg., specific activity 850 cpm per mg. Two crystallisations from aqueous methanol were performed. The specific activity of the final material was 22.7 cpm per mg. The results are

shown in Table IV.25. The mixed m.p. with authentic oestriol (m.p. 266-270°) was 262-268°. The spectrum of a solution of the final material in methanol was identical with that of authentic oestriol:

λ^{MeOH}_{Max} : 281 mμ (ε = 2007)

λ^{MeOH}_{Min} : 248-249 mμ (ε = 193).

The spectrum of a sulphuric acid chromogen of the final material (40.3 μg./3 ml. H_2SO_4) was identical with that of authentic oestriol (Section II):
Max. mμ (O.D.): 232 (0.521) [I]; 303 (0.281); 462 (0.200).
Min. mμ (O.D.): 264 (0.150); 348 (0.066).

h) Dehydroepiandrosterone (XIII).

Carrier dehydroepiandrosterone was purified by crystallisation from aqueous methanol to yield material exhibiting m.p. 140-149°.

Of the 1038 μg. carrier dehydroepiandrosterone originally added, 695 μg. was recovered of specific activity 9447 cpm per mg. Additional carrier dehydroepiandrosterone (18.03 mg.) was added to the radioactive material to give a total weight of 20.03 mg. of specific activity 332 cpm per mg. Two crystallisations were performed and the specific activity of the second crop of crystals was 58.9 cpm per mg. The

Table IV. 25. Specific activities, weights and m.p.'s of oestriol after successive crystallisations.
Incubation of slices with [4-^{14}C] progesterone.

Carrier added	980 µg.
Oestriol recovered	770 µg.; 20644 cpm per mg.
Additional carrier added	15.55 mg.
Total material	16.85 mg.; 850 cpm per mg.

		C R Y S T A L S			MOTHER LIQUOR	
Cryst.	Solvent	Wt. (mg.)	M.p.	Sp.act. (cpm per mg.)	Wt. (mg.)	Sp.act. (cpm per mg.)
1	Aqueous methanol	9.24	252-264°	68.1	6.95	2156
2	Aqueous methanol	2.69	263-268°	22.7	6.54	89

dehydroepiandrosterone was acetylated and after extraction the product was crystallised twice from aqueous methanol. The specific activity of the final material was 10.8 cpm per mg. free dehydroepiandrosterone. The m.p. of a mixture of the second crop of crystals with authentic dehydroepiandrosterone acetate (m.p. 163-165.5°) was 155-162°. The results are shown in Table IV.26.

i) Corticosterone (IV).

Carrier corticosterone was purified by crystallisation from aqueous methanol to give material of m.p. 170-176°.

Of the 950.6 µg. carrier corticosterone originally added, 217 µg. was recovered of specific activity 87,800 cpm per mg. Additional carrier corticosterone (22.26 mg.) was added to the radioactive material, giving a total weight of 22.78 mg. of specific activity 929 cpm per mg. Three crystallisations from aqueous methanol were performed. The specific activity of the third crystallisation was 495 cpm per mg. The material was acetylated and crystallised from aqueous methanol, the specific activity being 474 cpm per mg. free corticosterone (calculated). The results are shown in Table IV.27. A solution of the final material in methanol exhibited λ_{max} 241-243 mµ (ε = 16,320).

Table IV. 26. Specific activities, weights and m.p.'s of dehydroepiandrosterone after successive crystallisations, acetylation and subsequent crystallisations. Incubation of slices with [4-^{14}C] progesterone.

		CRYSTALS			MOTHER LIQUOR	
Cryst.	Solvent	Wt. (mg.)	M.p.	Sp.act.*	Wt. (mg.)	Sp.act.*
1	Aqueous methanol	9.58	129-140°	60.0	7.20	747
2	Chloro-form-methanol	6.74	114-130°	58.9	2.56	139
Acetylation Product		6.85	-	62.0		
1	Aqueous methanol	4.85	152-160°	24.4	1.66	181
2	Aqueous methanol	2.62	155-161°	10.8	-	-

* Cpm per mg. free dehydroepiandrosterone.

Table IV. 27. Specific activities, weights and m.p.'s of corticosterone after successive crystallisations, acetylation and crystallisation.
Incubation of slices with [4-^{14}C] progesterone.

Carrier added	950.6 µg.
Corticosterone recovered	217 µg.; 87800 cpm per mg.
Additional carrier added	22.26 mg.
Total material	22.78 mg.; 929 cpm per mg.

		C R Y S T A L S			MOTHER LIQUOR	
Cryst.	Solvent	Wt. (mg.)	M.p.	Sp.act.*	Wt. (mg.)	Sp.act.*
1	Aqueous methanol	16.51	168-175°	545	3.89	1631
2	Aqueous methanol	13.68	170-178°	464	2.60	769
3	Aqueous methanol	11.35	171-178°	495	1.92	532
Acetate						
1	Aqueous methanol	8.08	116-128°	474	4.75	398

* Cpm per mg. free corticosterone

The spectrum of a sulphuric acid chromogen of the final material (53.9 µg. acetate/3 ml. H_2SO_4) was identical with that of authentic corticosterone 21-monoacetate (Section II):

Max. mµ (O.D.): 239 (0.263); 286 (0.718); 338 (0.218); 376 (0.125); 472 (0.150).

Min. mµ (O.D.): 234 (0.261); 246 (0.259); 322 (0.197); 368 (0.118); 404 (0.090).

A second crystallisation of the acetate gave crystals of m.p. 137-142° and a mixed m.p. with authentic material (m.p. 123-130°) of 124-144°.

j) 11-Deoxycortisol (VII).

Carrier 11-deoxycortisol was purified by crystallisation from aqueous methanol to give material of m.p. 178-186°.

Of the 887.4 µg. carrier 11-deoxycortisol originally added, 418 µg. of specific activity 276,675 cpm per mg. was recovered. Additional carrier (22.12 mg.) was added to the radioactive material to give a total weight of 22.87 mg., specific activity 4674 cpm per mg. The material was crystallised from chloroform-methanol to yield material of specific activity 4783 cpm per mg. This material was acetylated and crystallised three times. The final crystals had a specific activity of 3836 cpm per mg. free 11-deoxycortisol (calculated). The results are shown in

Table IV.28. A solution of the final material in methanol exhibited λ_{max} 241-242 mμ (ε = 16,900). The spectrum of a sulphuric acid chromogen of the final material (41.4 μg. acetate/3 ml. H_2SO_4) was identical with that of authentic 11-deoxycortisol monoacetate (Section II):

Max. mμ (O.D.): 210 (0.302); 240 (0.310); 287 (0.668); 334 (0.228); 494 (0.095) [I]; 538 (0.1575).

Min. mμ (O.D.): 224 (0.280); 250 (0.262); 323 (0.219); 404 (0.041).

A mixed m.p. with authentic 11-deoxycortisol 21-monoacetate (m.p. 208-214°) was 210-222°.

Table IV. 28. Specific activities, weights and m.p.'s of 11-deoxycortisol after crystallisation, acetylation and subsequent crystallisations.
Incubation of slices with [4-^{14}C] progesterone.

Carrier added	887.4 µg.
11-Deoxycortisol recovered	418 µg.; 276675 cpm per mg.
Additional carrier added	22.12 mg.
Total material	22.87 mg. ; 4674 cpm per mg.

		C R Y S T A L S			MOTHER LIQUOR	
Cryst.	Solvent	Wt. (mg.)	M.p.	Sp.act.*	Wt. (mg.)	Sp.act.*
1	Chloroform-methanol	12.15	168-178°	4783	10.68	4390
Acetate						
1	Aqueous methanol	12.57	210-218°	3555	0.96	3684
2	Chloroform-methanol	11.04	220-224°	3801	1.14	4668
3	Aqueous methanol	9.61	214-220°	3836	-	-

* Cpm per mg. free 11-deoxycortisol.

(v) Incubation of homogenate with [4-^{14}C] progesterone.

In the incubation of the homogenate of the adrenal tumour with [4-^{14}C] progesterone, some of the steroids were assayed for radioactivity. The results are shown in Table IV.29. The radiochemical purity of these compounds was not investigated further.

Table IV. 29. Specific activities, weights and m.p.'s of steroids recovered.
Incubation of homogenate with [4-^{14}C] progesterone.

Steroid	Carrier added (µg.)	Steroid recovered (µg.)	Sp.act. (cpm per mg.)
Cortisol	997	638	150517
Androstenedione	1026	961	35609
Oestrone	1013	331	9441
Oestradiol-17β	1005	813	1562
Oestriol	980	881	62509
17α-Hydroxy-progesterone	751	610	20320
Progesterone	966	841	35826

4. Discussion

The criterion of radiochemical purity of the steroids studied was constancy of specific activity of a crystalline compound through successive crystallisations, the formation of a derivative and subsequent crystallisations, or constancy of specific activity of a compound after two or more successive paper chromatograms. On this basis the following steroids isolated from the incubation of feminising adrenal tumour slices with [4-^{14}C] progesterone have been shown to be radiochemically pure: cortisol (VIII), androstenedione (XIV), 17α-hydroxyprogesterone (VI), corticosterone (IV) and 11-deoxycortisol (VII). In the same incubation the following steroids could not be shown to be radioactive: oestrone (XIX), oestradiol (XX), oestriol (XXI) and dehydroepiandrosterone (XIII). By the second criterion, progesterone (II) isolated from the incubation of tumour slices with [1-^{14}C] acetate, has been shown to be radiochemically pure in all three experiments, that is, in the presence of ACTH or HMG and also in the absence of any trophic hormone. Cortisol (VIII) and androstenedione (XIV) were radioactive in the incubation with ACTH. 17α-Hydroxyprogesterone (VI) was radioactive in the experiment in which HMG was present.

By employing the procedure of crystallisation, formation of a derivative and the subsequent crystallisation, rigorous purification of the steroids studied in the incubation of tissue slices with [4-^{14}C] progesterone has been achieved. In the incubations with [1-^{14}C] acetate, in most cases too little radioactivity was found associated with the steroids to permit use of the same technique. These steroids were therefore further purified by paper chromatography. Oestrone (XIX) and oestriol (XXI) were acetylated where further study of these two steroids was merited by the amount of radioactivity present. However in no case were they shown to be radioactive. Androstenedione (XIV), 17α-hydroxyprogesterone (VI) and progesterone (II) were purified by subjecting them to acetylation conditions. Unfortunately insufficient 17α-hydroxyprogesterone (VI) remained in the experiment with ACTH after acetylation conditions (which do not acetylate 17α-hydroxyprogesterone) to permit further study. Cortisol (VIII) was found to be radioactive after the incubation of tissue slices with ACTH. However it is doubtful whether the radioactivity of this cortisol (VIII) is in fact all localised in cortisol (VIII) itself. More rigorous purification either by acetylation as in the incubation with HMG or by crystallisation as in the incubation without trophic hormone would have been preferable.

The feminising adrenal tumour slices have been shown to possess the enzymes necessary for the synthesis of at least certain steroids from acetate. Progesterone (II) was radioactive in all three experiments in which the radioactive precursor was [1-^{14}C] acetate. In addition, enzymes for the synthesis of androstenedione (XIV), 17α-hydroxyprogesterone (VI) and possibly cortisol (VIII) were present although the evidence for this was obtained only when trophic hormones were present. The data obtained in this study does not permit the suggestion of a mechanism of action of the trophic hormones. The minimum incorporation of [1-^{14}C] acetate into progesterone (II) in the presence of ACTH, HMG and with no trophic hormone was 0.0011%, 0.00018% and 0.00028% respectively. Thus ACTH increased the conversion of acetate to progesterone (II) by a factor of 6.1 whereas HMG increased it by a factor of 1.55. At this point the response to ACTH infusion of the patient from whom this tumour was obtained should be considered. The level of plasma 17-hydroxysteroids showed only a slight response to ACTH infusion, suggesting either that the tumour is not responsive to stimulation by exogenous ACTH or that it is already under maximum stimulation. The latter is an unlikely occurrence since the above normal concentration of circulating 17-hydroxysteroids would normally depress ACTH output. In the <u>in vitro</u> studies it has been shown that ACTH can stimulate activity, at least of the synthesis

of progesterone (II) from acetate.

The presence of enzymes for the subsequent hydroxylation of progesterone (II) have been clearly demonstrated in the studies with [4-^{14}C] progesterone. The minimum conversion of progesterone (II) to 17α-hydroxyprogesterone (VI) was 0.022%. Subsequent hydroxylations at C-21 followed by that at C-11 yielded 11-deoxycortisol (VII) (7.8% minimum conversion from progesterone (II)) and cortisol (VIII) (2.3% minimum conversion from progesterone (II)) respectively. Hydroxylations at C-21 and C-11 were also shown by the presence of radioactive corticosterone (IV) (2.0% minimum conversion). The high rate of synthesis of 11-deoxycortisol (VII) compared with that of cortisol (VIII) is interesting. No study of the deoxycorticosterone (III) in these experiments was made, the activity of which might have shed some light on the problem of the activity of 11β-hydroxylase in this tumour. Whether the high activity of 11-deoxycortisol (VII) found *in vitro* is of significance clinically is not known.

It has been clearly shown that the tumour possesses enzymes for the synthesis of androstenedione (XIV) from progesterone (II). The minimum conversion was 0.48%. The finding of no radioactivity in dehydroepiandrosterone (XIII) is not surprising as it has been shown that dehydroepiandrosterone (XIII) is derived directly from pregnenolone (XI), rather than by a pathway involving progesterone (II) (Goldstein *et al.*, 1958).

No synthesis of oestrogens by the tumour slices could be demonstrated. This result is in contrast to the evidence presented by Baggett and his coworkers (1959) who demonstrated the *in vitro* conversion of [3-^{14}C] testosterone (XVI) to oestrone (XIX) and oestradiol-17β (XX) but not to oestriol (XXI) by slices of a feminising adrenal carcinoma from a 66-year-old male. The hypothesis that the tumour rather than another tissue synthesises the large amount of oestrogen, as evidenced by the high urinary values, is supported by the identification of oestrone (XIX) in a feminising adrenal carcinoma (Romanelli, *et al.*, 1960). The dramatic fall in urinary oestrogens following extirpation of the tumour also supports this hypothesis. One explanation of the failure to demonstrate oestrogen biosynthesis in the present study is the small weight of tissue (3.1-3.7 g.) used in the incubations relative to the weight of the total tumour (1100 g.).

The pathway of oestrogen biosynthesis has been shown to exist in this tumour as far as the C-19 steroid, androstenedione (XIV). It is possible that if a portion of the tumour had been incubated with ^{14}C-androstenedione (XIV) of higher specific activity than that isolated, or with ^{14}C-testosterone (XIV), radioactive oestrogens would have been isolated.

Section V: Discussion

The purpose of the present research was to study steroid hormone biosynthesis in pathological tissues using an *in vitro* technique. Such techniques permit close control of the experimental conditions, using tissue which is isolated from the influence of other organs, and thus allowing comparisons to be made from one experiment to another.

The *in vitro* technique which permits an organ to function in a manner most closely related to that *in vivo* is the method of organ perfusion. Indeed Vogt (1951), using this technique, claims that the output of corticosteroids by the isolated adrenal gland of the dog is approximately that of normal unstressed dogs. It is interesting also to recall that the first elucidation of the pathway of steroid hormone biosynthesis was achieved using results obtained from perfusion studies of the cow adrenal. (Hechter *et al.*, 1951). This technique however demands the whole intact tissue, which is not always available in specimens from human subjects.

Perhaps the most commonly used *in vitro* technique in steroid biosynthetic experimentation has been that of incubating slices of an endocrine tissue in a physiological medium. The media employed usually reflect the ionic composition of the blood of the

animal being studied. In the saline media, no protein comparable to that of the naturally circulating plasma proteins is present and this represents a considerable loss in properties. Another factor contributing to an apparent low activity in a tissue may be the mechanical difficulties of obtaining tissue slices. At present, it is difficult to achieve uniform slices, especially with soft tissues. The use of tissue minces is subject to this same criticism of difficulty of duplication. In addition the loss of organ structure, that is the juxtaposition of particular cell types, may contribute to misleading data.

With homogenates also there is some difficulty in the interpretation of data. Inherent in the homogenising technique is the destruction of cellular structures with subsequent dilution of the cellular components. Thus it must be considered that the failure to demonstrate enzymic activity may have been caused by a failure of concentration, either of the concentration of all the necessary cofactors or of the concentration of a substrate at the appropriate site of action of an enzyme. Differential centrifugation of homogenates yields subcellular fractions which, although useful in the study of isolated enzymes, are subject to the same limitations as homogenates of whole tissue and are therefore of restricted use when a complex reaction sequence is being studied.

The *in vitro* technique most commonly used in the present study has been the incubation of tissue slices in a physiological medium. In this way the pathological tissues used could be studied without the interactions from other tissues prevalent *in vivo*. The tissue slice technique was chosen firstly because it was hoped that in this way the conditions were more nearly comparable to the *in vivo* state of the tissue than was a homogenate. Secondly slices from the same tissue could be studied under a variety of conditions, an experimental design which could not have been realised with organ perfusion, even had it been possible to obtain the whole intact tissue. Only with the technique of tissue slice incubation was it possible to study the biosynthesis of steroid hormones by a feminising adrenal tumour, from both [1-^{14}C] acetate and [4-^{14}C] progesterone in the presence of different trophic factors, corticotrophin and human menopausal gonadotrophin.

The tissues studied were, first, ovaries obtained from women with the Stein-Leventhal syndrome and, second, a feminising adrenal tumour from a 39-year-old man. The synthesis of radioactive oestrone (XIX), oestradiol (XX), testosterone (XVI), 17α-hydroxyprogesterone (VI), androstenedione (XIV) and progesterone (II) from sodium [1-^{14}C] acetate by tissue slices of Stein-Leventhal ovaries has been demonstrated.

Using tissue slices of a feminising adrenal tumour, the transformation of [4-^{14}C] progesterone to radioactive cortisol (VIII), androstenedione (XIV), 17α-hydroxyprogesterone (VI), corticosterone (IV) and 11-deoxycortisol (VII) has been shown. In three other incubations of the same tissue, one in the presence of corticotrophin (ACTH), another in the presence of human menopausal gonadotrophin (HMG) and the third in the absence of any trophic factor, radioactive progesterone (II) was synthesised from sodium [1-^{14}C] acetate. In the case where ACTH was present both ^{14}C-cortisol (VIII) and ^{14}C-androstenedione (XIV) were isolated. Labelled 17α-hydroxyprogesterone (VI) was isolated from the incubation mixture which had contained HMG. Discussion of the significance of these results has already been included in Sections III and IV respectively. The results are in agreement with the concept of the same general pattern of steroid hormone biosynthesis in all steroidogenic endocrine tissues outlined in Section I.

However, in conclusion, the significance of results obtained from <u>in vitro</u> studies of pathological tissues should be examined. In congenital adrenal hyperplasia there is a defect in the C-21 hydroxylating system which produces cortisol (VIII) from 17α-hydroxyprogesterone (VI) via 11-deoxycortisol (VII) (Dorfman, 1955; Bongiovanni, 1958) and sometimes in the C-11 hydroxylating system (Bongiovanni and Eberlein, 1956).

Incubation studies *in vitro* (Bongiovanni, 1958; O'Donnell *et al.*, 1960) have demonstrated a predominance of the 17α-hydroxylase system in this type of adrenal tissue. This observation is in agreement with the report that excessive amounts of pregnane-3α:17α:20α-triol (XXIX), the main degradation product of the 17α-hydroxyprogesterone (VI), were found in the urine of patients with this syndrome. (Fukushima and Gallagher, 1957). Thus the *in vitro* activity of the adrenal offers a solution of the *in vivo* biochemical manifestations of the disorder.

In this way, an *in vitro* technique may make apparent an enzyme deficiency in the tissue being studied. However in such a complex system as the endocrine glands with their central control in the region of the hypothalamus, via the anterior pituitary, theoretically an endocrine disorder may result from an upset of the endocrine balance at any stage in the system. As an example of such interaction, clinical hypoadrenalism is present in both Simmonds's disease and Addison's disease. In Simmonds's disease the lack of output of steroid hormones by the adrenals is due to a lack of stimulation by corticotrophin, produced by the pituitary gland. In Addison's disease on the other hand, the adrenals fail to respond to stimulation.

It must be concluded therefore that data regarding the biosynthesis of steroid hormones by

pathological tissues using an *in vitro* technique should be interpreted with caution, especially when attempting to extrapolate to *in vivo* conditions. However an extensive comparison of results obtained from well-defined *in vitro* studies of both abnormal and normal tissues would provide data which might clarify somewhat the problem of the causes of endocrine disorders.

Section VI : Summary

I. Slices of Stein-Levanthal ovaries were incubated with sodium [1-^{14}C] acetate.

II. Slices of a feminising adrenal tumour were incubated with [4-^{14}C] progesterone. Slices of the same tissue were incubated with sodium [1-^{14}C] acetate in the presence of corticotrophin, human menopausal gonadotrophin, and in the absence of any trophic factor.

III. The radioactive steroids elaborated by these tissues were studied using reverse isotope dilution techniques and purified by paper chromatography and crystallisation to constant specific activity. The steroids were further purified by the formation of derivatives and the subsequent hydrolysis of these derivatives where this was possible.

IV. The biosynthesis from [1-^{14}C] acetate by slices of Stein-Levanthal ovaries of the following steroids was established: oestrone (XIX), oestradiol (XX), testosterone (XVI), 17α-hydroxy-progesterone (VI), androstenedione (XIV) and progesterone (II).

V. The conversion of [4-^{14}C] progesterone by slices of a feminising adrenal tumour to the following steroids was established: cortisol (VIII),

androstenedione (XIV), 17α-hydroxyprogesterone (VI), corticosterone (IV) and 11-deoxycortisol (VII). Proof has been given for the synthesis of ^{14}C-progesterone from [1-^{14}C] acetate by slices of this tissue incubated in the presence of corticotrophin (ACTH), human menopausal gonadotrophin (HMG), and in the absence of any trophic factor. From the incubation in which ACTH was present, ^{14}C-cortisol (VIII) and ^{14}C-androstenedione were isolated. From the incubation in which HMG was present, ^{14}C-17α-hydroxyprogesterone (VI) was isolated.

VI. The significance of these results has been discussed in relation to the tissue under study and also to a possible correlation with <u>in vivo</u> conditions.

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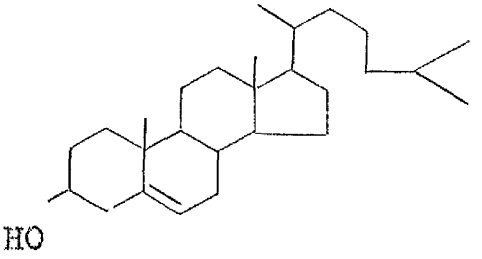
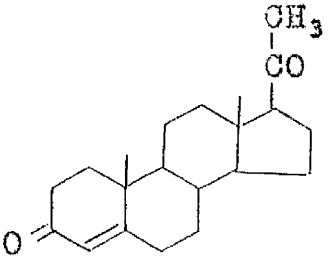
Section VIII : Steroids:nomenclature and trivial name.

I Cholesterol



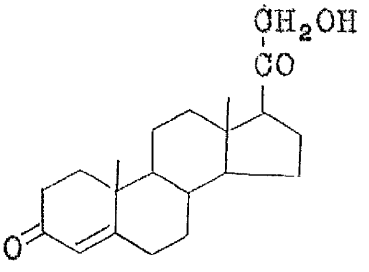
II Pregn-4-ene-3:20-dione

(progesterone)



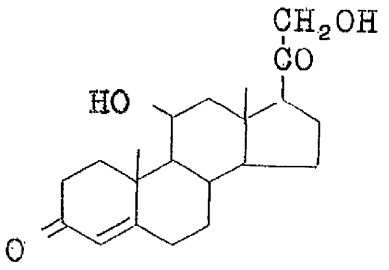
III 21-Hydroxypregn-4-ene-3:20-dione

(deoxycorticosterone)



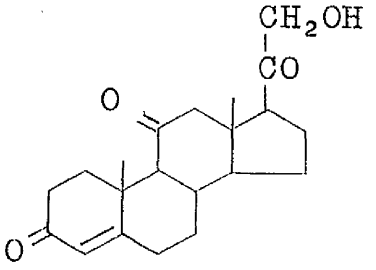
IV 11β:21-dihydroxypregn-4-ene-3:20-dione

(corticosterone)



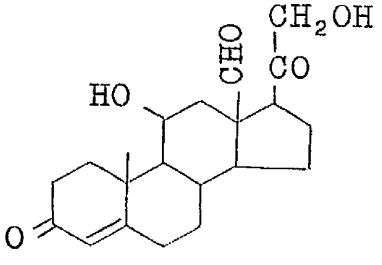
IVb 21-hydroxypregn-4-ene-3:11:20-trione

(11-dehydrocorticosterone)



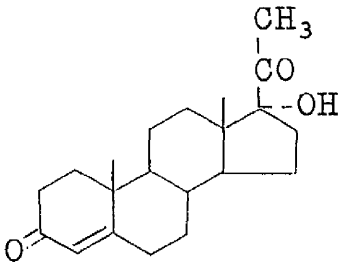
V 11β:21-dihydroxy-3:20-dioxopregn-4-en-18-al

(aldosterone)



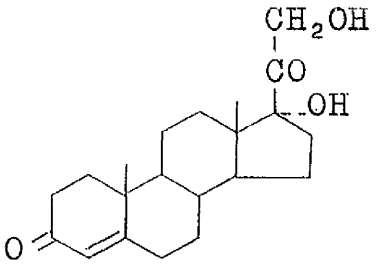
VI 17α-Hydroxypregn-4-ene-3:20-dione

(17α-hydroxyprogesterone)



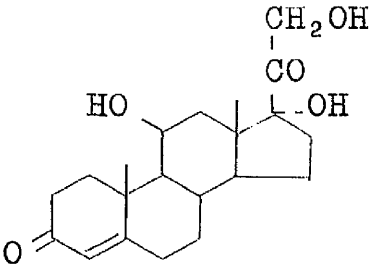
VII 17α:21-Dihydroxypregn-4-ene-3:20-dione

(11-deoxycortisol)



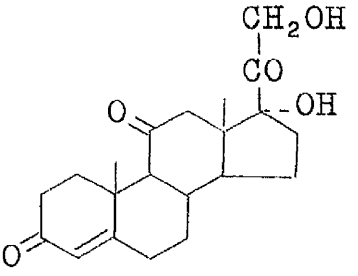
VIII 11β:17α:21-Trihydroxypregn-4-ene-3:20-dione

(cortisol)



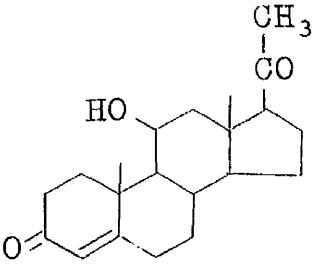
IX 17α:21-dihydroxypregn-4-ene-3:11:20-trione

(cortisone)



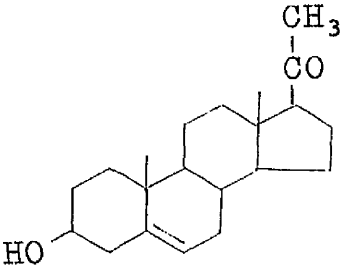
X 11β-Hydroxypregn-4-ene-3:20-dione

(11β-hydroxyprogesterone)

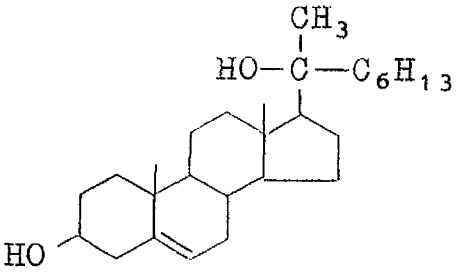


XI 3β-Hydroxypregn-5-en-20-one

(pregnenolone)

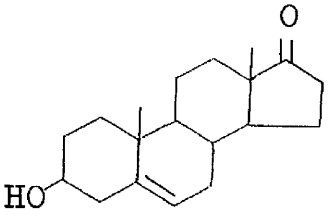


XII 20β-Hydroxycholesterol



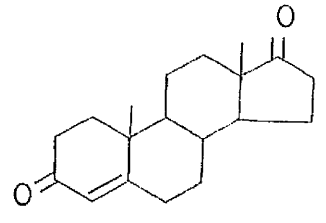
XIII 3β-Hydroxyandrost-5-en-17-one

(dehydroepiandrosterone)



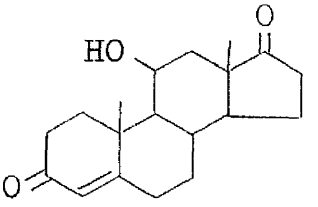
XIV Androst-4-ene-3:17-dione

(androstenedione)



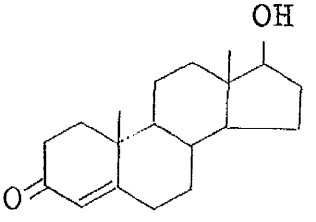
XV 11β-Hydroxyandrost-4-ene-3:17-dione

(11β-hydroxyandrostenedione)



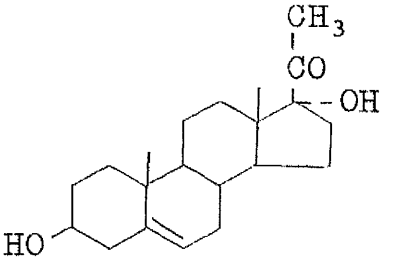
XVI 17β-Hydroxyandrost-4-en-3-one

(testosterone)



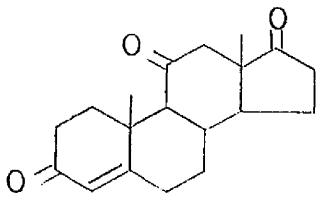
XVII 3β:17α-Dihydroxypregn-5-en-20-one

(17α-hydroxypregnenolone)



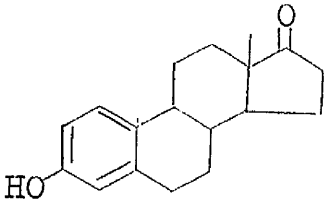
XVIII Androst-4-ene-3:11:17-trione

(adrenosterone)



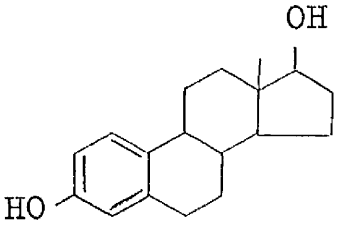
XIX 3-Hydroxyoestra-1:3:5(10)-trien-17-one

(oestrone)



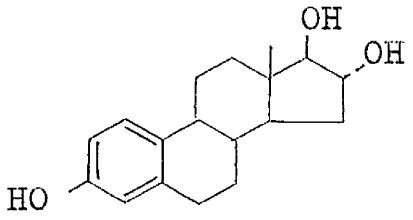
XX Oestra-1:3:5(10)-triene-3:17β-diol

(oestradiol-17β)

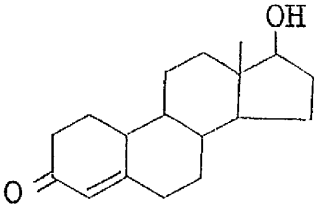


XXI Oestra-1:3:5(10)-triene-3:16α:17β-triol

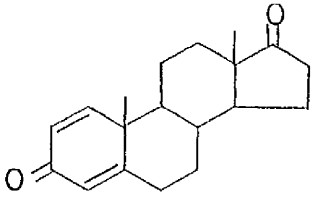
(oestriol)



XXII 19-Nortestosterone

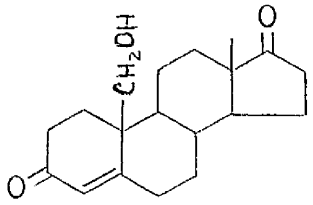


XXIII Androst-1:4-diene-3:17-dione



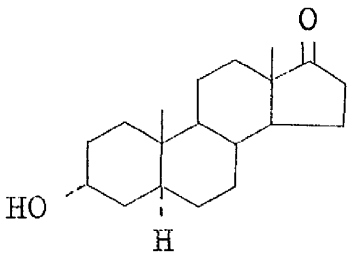
XXIV 19-Hydroxyandrost-4-ene-3:17-dione

(19-hydroxyandrostenedione)



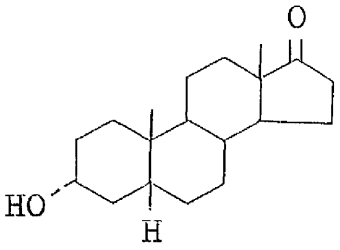
XXV 3α-Hydroxy-5α-androstan-17-one

(androsterone)



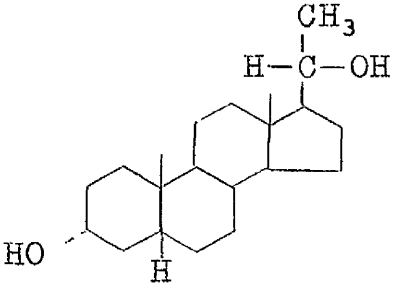
XXVI 3α-hydroxy-5β-androstan-17-one

(aetiocholanolone)



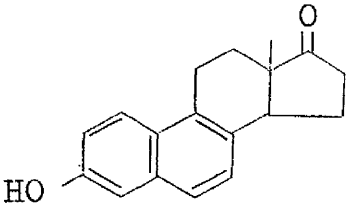
XXVII Pregnane-3α:20α-diol

(pregnanediol)

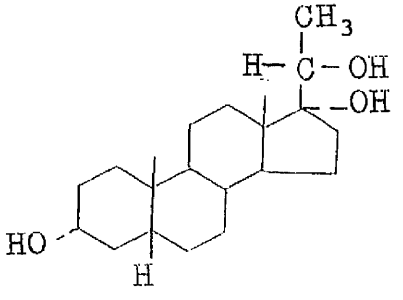


XXVIII 3-Hydroxyoestra-1:3:5(10):6:8-pentaen-17-one

(equilenin)



XXIX Pregnane-3α:17α:20α-triol



A Study on Steroid Hormone Biosynthesis

by Jean Gray Birnie, B.Sc.

In recent years the pathways of steroid hormone biosynthesis have received a great deal of attention. An increasing amount of evidence for the concept of a general pathway of steroid hormone biosynthesis in all steroidogenic endocrine tissues has accumulated from the published results of studies in various tissues of a number of species. The object of the present study was to investigate the steroidogenic capacity of two types of pathological tissue, namely, ovaries from women with the Stein-Levanthal syndrome and a feminising adrenal tumour from a 39-year-old man.

Slices of these tissues, obtained from patients at surgery, were incubated at 37° C for 3 hours with sodium [1-^{14}C] acetate or [4-^{14}C] progesterone in Krebs-Ringer phosphate buffer, pH 7.4, supplemented with glucose, fumarate and nicotinamide. The incorporation of the isotope into various steroid hormones was studied by means of the reverse isotope dilution technique. Steroids were purified either by paper chromatography or by crystallisation and preparation of derivatives. The criterion of radiochemical purity was taken as constancy of specific

activity measured after two successive paper chromatograms or crystallisations.

It has been found that, when slices of Stein-Levanthal ovaries were incubated with ^{14}C-acetate, the isotope was incorporated into oestrone, oestradiol and a substance which was similar to, but not identical with, oestriol. In addition, it has been shown that this ovarian tissue synthesised progesterone, androstenedione, testosterone and 17α-hydroxyprogesterone from ^{14}C-acetate. The presence of human menopausal gonadotrophin in the incubation medium decreased the amount of ^{14}C-acetate incorporated into androstenedione and increased the amount incorporated into progesterone, testosterone and 17α-hydroxyprogesterone.

It has been established that when slices of the feminising adrenal tumour were incubated, ^{14}C-progesterone was converted to cortisol, corticosterone, 11-deoxycortisol, 17α-hydroxyprogesterone and androstenedione. In addition, the biosynthesis of ^{14}C-progesterone from ^{14}C-acetate by this tumour tissue has been demonstrated. The presence of corticotrophin and human menopausal gonadotrophin in the incubation medium increased this incorporation of ^{14}C-acetate into progesterone by factors of 6.1 and 1.55 respectively. It has been shown that, when corticotrophin was present, androstenedione and cortisol were synthesised from ^{14}C-acetate. In the incubation in which human

menopausal gonadotrophin was included, 17α-hydroxy-progesterone was synthesised. In no incubation could the biosynthesis of oestrone, oestradiol or oestriol by the adrenal tumour be demonstrated.

The foregoing results agree with the concept of a general pathway of steroid hormone biosynthesis in all steroidogenic tissues. Experiments of this type may illuminate an enzyme deficiency within a pathological tissue and in this way, elucidate the clinical biochemical manifestations associated with the resulting disease.