STUDIES ON THE ORIGIN OF URINARY STEROIDS

by

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TABLE OF CONTENTS

Page

ABBREVIATIONS AND TRIVIAL NAMES.

The following abbreviations and trivial names are used in this thesis:

dehydroisoandrosterone - 3β-hydroxy-Δ^5-androsten-17-one

androsterone - 3α-hydroxyandrostan-17-one

etiocholanolone - 3α-hydroxyetiocholan-17-one

11-ketoetiocholanolone - 3α-hydroxyetiocholane-11,17-dione

11β-hydroxyetiocholanolone - 3α,11β-dihydroxyetiocholan-17-one

11β-hydroxyandrosterone - 3α,11β-dihydroxyandrostan-17-one

isoandrosterone - 3β-hydroxyandrostan-17-one

11-ketoandrosterone - 3α-hydroxyandrostane-11,17-dione

11β-hydroxyandrostenedione - 11β-hydroxy-Δ^4-androstene-3,17-dione

isoetiocholanolone - 3β-hydroxyetiocholan-17-one

androstenedione - Δ^4-androstene-3,17-dione

16α-hydroxydehydroisoandrosterone - 3β,16α-dihydroxy-Δ^5-androsten-17-one

androstanedione - androstane-3,17-dione

etiocholanedione - etiocholane-3,17-dione

7-ketodehydroisoandrosterone - 3β-hydroxy-Δ^5-androstene-7,17-dione

7α-hydroxydehydroisoandrosterone - 3β,7α-dihydroxy-Δ^5-androsten-17-one

7α,16α-dihydroxydehydroisoandrosterone - 3β,7α,16α-trihy-

droxy-Δ^5-androsten-17-one

16α-hydroxy-7-ketodehydroisoandrosterone - ,3β,16α-dihydroxy-Δ^5-androstene-7,17-dione

16α-hydroxyandrosterone - 3α,16α-dihydroxyandrostan-17-one

18-hydroxyandrosterone - 3α,18-dihydroxyetiocholan-17-one

18-hydroxyetiocholanolone - 3α,18-dihydroxyetiocholan-17-one

6β-hydroxyandrostenedione - 6β-hydroxy-Δ^4-androstene-3,17-dione

pregnenolone - 3β-hydroxy-Δ^5-pregnen-20-one

17α-hydroxypregnenolone - 3β,17α-dihydroxy-Δ^5-pregnen-20-one

hydrocortisone - 11β,17α,21-trihydroxy-Δ^4-pregnene-3,20-dione

cortisone - 17α,21-dihydroxy-Δ^4-pregnene-3,11,20-dione

progesterone - Δ^4-pregnene-3,20-dione

17α-hydroxyprogesterone - 17α-hydroxy-Δ^4-pregnene-3,20-dione

11-desoxyhydrocortisone - 17α,21-dihydroxy-Δ^4-pregnene-3,20-dione

testosterone - 17β-hydroxy-Δ^4-androsten-3-one

cholesterol - 3β-hydroxy-Δ^5-cholestene

tetrahydro-11-desoxyhydrocortisone - 3α,17α,21-trihydroxy-pregnan-20-one

21-desoxycortisone - 17α-hydroxy-Δ^4-pregnene-3,11-20-trione

androstanediol - 3α,17β-dihydroxyandrostane

etiocholanediol - 3α,17β-dihydroxyetiocholane

pregnenetriol - 3β,17α,20α-trihydroxy-Δ^5-pregnene

Δ^5-androstenediol - 3β,17β-dihydroxy-Δ^5-androstene

Δ^5-pregnenediol - 3β,20α-dihydroxy-Δ^5-pregnene

pregnanetriol - 3α,17α,20α-trihydroxypregnane

pregnanediol - 3α,20α-dihydroxypregnane

dexamethazone - 16α-methyl -9α-fluoro-11β,17α,21-trihydroxy-$\Delta^{1,4}$-pregnadiene-3,20-dione

11β-hydroxyisoandrosterone - 3β,11β-dihydroxyandrostan-17-one

ACTH - adrenocorticotrophic hormone

HCG -- human chorionic gonadotrophin

μg - microgram

mg - milligram

gm - gram

μc - microcurie

cpm - counts per minute

ml - milliliter

PART I.

THE EFFECT OF DEHYDROISOANDROSTERONE ON THE "IN VIVO" CONVERSION OF 17α-HYDROXYPREGNENOLONE TO URINARY 11-DESOXY-17-KETOSTEROIDS.

INTRODUCTION

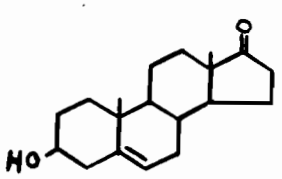
A large number of 17-ketosteroids have been found in the urine since the first one was isolated almost thirty-five years ago. Butenandt and his co-workers in 1931 (1) first isolated a crystalline steroid with androgenic activity from a chloroform extract of normal male urine which had been hydrolysed with acid and subsequently identified it as androsterone in 1934 (2). In the same year, these authors described the isolation of dehydroisoandrosterone from urine. (3)..

In 1938 Butler and Marrian (4) reported the isolation of two other 17-ketosteroids, etiocholanolone and isoandrosterone, from a large quantity of unhydrolysed urine excreted by a woman with an adrenocortical tumour. The isolation of etiocholanolone from the urine of a normal male subject was reported by Callow (5) in 1939. Dehydroisoandrosterone, androsterone and etiocholanolone, which differ in rings A and B of the molecule, (Fig. 1), are the principal 11-desoxy-17-ketosteroids excreted in the urine by normal subjects. In comparison to these three, isoandrosterone is normally excreted in very small quantities. Androsterone and dehydroisoandrosterone were first isolated from the urine of normal women by Callow and Callow in 1938 (6); the quantities were similar to those found in male urine. In 1939, Callow and Callow (7) also reported that etiocholanolone was present in the urine of normal females in amounts similar to those found in urine from normal males.

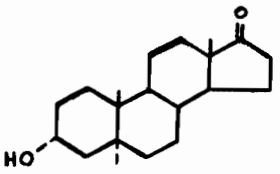
Since the isolations of these 17-ketosteroids, a large number of

Figure 1.

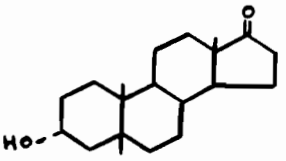
THE MOST ABUNDANT 17-KETOSTEROIDS ISOLATED FROM HUMAN URINE.



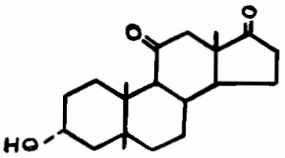
Dehydroisoandrosterone



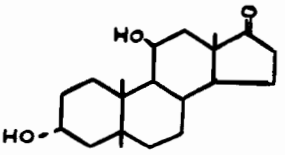
Androsterone



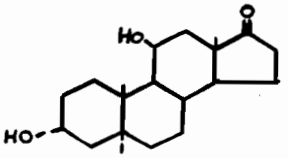
Etiocholanolone



11-Ketoetiocholanolone



11β-Hydroxyetiocholanolone



11β-Hydroxyandrosterone

other steroids with a 17-ketone function have been found in human urine. Of these other urinary steroids, the 11-oxy-17-ketosteroids were quantitatively the most important. Mason (8, 9) and Mason and Kepler (10) identified 11β-hydroxyandrosterone in the urine of subjects with adrenocortical tumors or hyperplasia and in the urine of normal subjects. Lieberman and his co-workers found 11-ketoetiocholanolone (11, 12) in the urine of normal men and women and patients with various diseases. These authors (13, 14, 15) also found 11-ketoandrosterone in the urine of normal and diseased subjects and 11β-hydroxyetiocholanolone in the urine of the diseased but not the normal subjects. Kemp et al (16) identified 11β-hydroxyisoandrosterone in the urine of a patient receiving large quantities of hydrocortisone. One of the precursors of the 11-oxygenated steroids just discussed, 11β-hydroxy-Δ^4-androstenedione, has itself been isolated by Salamon and Dobriner (17) from the urine of subjects receiving large quantities of adrenocorticotropin (ACTH).

During the course of their studies, Lieberman and his co-workers isolated several urinary 11-desoxy-17-ketosteroids which had not been described previously. These include isoetiocholanolone (15) from the urine of normal males and non-pregnant females, pregnant females and diseased subjects and androstanedione (12) from the urine of normal males and females. Etiocholanedione (12) was found in the urine of normal males and females and subjects with hypertension and Cushing's syndrome and Δ^4-androstenedione (12) was identified in urine of a patient with adrenocortical hyperplasia. Miller et al (18) have also reported

the isolation of androstenediane from the urine of a woman with adrenocortical carcinoma.

A number of compounds related to dehydroisoandrosterone in that they have the 3β-hydroxy-Δ^5 structure in rings A and B have been isolated from urine. Fotherby et al (19) isolated 16α-hydroxydehydroisoandrosterone from the urine of normal subjects. These authors were also able to isolate 16α-hydroxydehydroisoandrosterone from the urine of adrenalectomized and ovariectomized woman following the intramuscular administration of dehydroisoandrosterone, thus showing that the 16-hydroxylated compound was a metabolite of the latter steroid. Fukushima et al (20) isolated 7-ketodehydroisoandrosterone from the urine of subjects with adrenocortical tumors and hyperplasia and also from the urine of normal subjects. Schneider and Lewbart (21) found 7α-hydroxydehydroisoandrosterone in the urine following the oral administration of large quantities of dehydroisoandrosterone. Okada et al (22) isolated 7α,16α-dihydroxydehydroisoandrosterone, 16α-hydroxy-7-ketodehydroisoandrosterone, 7α-hydroxydehydroisoandrosterone and 16α-hydroxydehydroisoandrosterone from the urine of a patient with adrenocortical carcinoma.

Hydroxylated derivatives of androsterone and etiocholanolone which have been identified in urine extracts include 16α-hydroxyandrosterone (21) found after the oral ingestion of large quantities of androsterone. Also, 18-hydroxyetiocholanolone (23) was found in the urine of a patient with an arrhenoblastoma who was excreting 100 mg. of etiocholanolone per day and in the urine of subjects given large amounts of testosterone and etiocholanolone. Following the intramuscular

administration of large quantities of androsterone 18-hydroxyandrosterone (23) was isolated from the urine. Recently, 6β-hydroxyandrostenedione has been isolated from the urine of a patient with adrenal carcinoma (24).

QUANTITATION OF URINARY 17-KETOSTEROIDS

The urinary excretions of individual 17-ketosteroids for 8 normal males and 13 normal females as reported by Jailer et al (25) and Vande Wiele and Lieberman (26) are summarized in Table I.

It can be seen that there is no great difference in the total excretion of these 17-ketosteroids by males and females. The significant difference between the two groups is in the quantities of the individual steroids excreted. The range of values for the two groups overlap extensively. This similarity in total excretion had been noted earlier by Dingemanse et al (27) who had found that the two sexes differed only in that males excreted a larger quantity of 3β-hydroxy-17-ketosteroids. This is also noted in the values presented in Table I. It should be noted also from Table I that the 11-desoxy-17-ketosteroids make up approximately 80 per cent of the total identifiable 17-ketosteroids and dehydroisoandrosterone makes up approximately 25 per cent of the total 11-desoxy-17-ketosteroid excretion.

It has been noted by a number of workers that age influences the quantities of 17-ketosteroids excreted in the urine. Kappas and Gallagher studied the α-ketosteroid excretion patterns in male (28) and female (29) adults and found that the 11-oxy-17-ketosteroids do not change with age but that the urinary levels of 11--desoxy-17-ketosteroids

TABLE I

Urinary levels of 17-ketosteroids for normal males and females.

(mg./24 hours)

17-ketosteroid	Males		Females	
	Average	Range	Average	Range
Total identified 17-ketosteroids	13.7	5.5 - 20.5	7.7	4 - 18
Dehydroisoandrosterone	2.7	0.1 - 8.8	1.8	0 - 6*
Isoandrosterone	1.7	0.3 - 3.0		
Androsterone	3.6	1.8 - 5.7	2.0	0.9 - 3.9
Etiocholanolone	3.1	0.8 - 6.5	2.0	0.6 - 3.8
11β-hydroxyandrosterone	1.5	0.6 - 3.5	1.0	0.5 - 1.7
11β-hydroxyetiocholanolone	0.5	0.2 - 0.9	0.5	0 - 1.5
11-ketoetiocholanolone	0.6	0.1 - 2.9	0.5	0 - 1.0

*Dehydroisoandrosterone and isoandrosterone were not determined individually here.

decrease with age. Beas et al (30) fractionated the 17-ketosteroids excreted by both children and adults. They could find no dehydroisoandrosterone in urine from normal girls and boys. Children were found to excrete more 11-oxy-17-ketosteroids than 11-desoxy-17-ketosteroids while the reverse was found for the adults (all the children studied were 7 years of age). Male children excreted twice as much 11β-hydroxyandrosterone as female children.

A number of investigators have reported that the amount of 17-ketosteroids excreted varies considerably from day to day. An extreme example of this is the daily fluctuation found in a subject with Cushing's syndrome (31) whose output varied from 5 to 45 mg. per day.

THE ORIGIN OF THE URINARY 17-KETOSTEROIDS

Shortly after the significance of the urinary 17-ketosteroids became apparent, many investigators undertook to determine the origin of these steroids. It was realized early that the precursors of the urinary 17-ketosteroids were secreted by the gonads and the adrenal glands. This early knowledge was derived from studies of the urinary steroids in subjects with hyper-, or hypo-function of the adrenals, ovaries or testes.

Hirschmann (32, 33) found that the amounts of dehydroisoandrosterone, androsterone and etiocholanolone in the urine of ovariectomized women were not lower than those of normal controls. From these studies he concluded that the adrenals were the major source of the precursors of the urinary 17-ketosteroids in normal females.

In 1940 Callow et al (34), reported on the excretion of 17-ketosteroids in the urine of subjects with gonadal and adrenocortical deficiencies, The average amounts of 17-ketosteroids in the urine of 11 enuchs and 18 ovariectomized women were in the lower part of the range of results obtained with normal subjects. The excretion of 17-ketosteroids was generally low in cases of Addison's disease. These results implicated both the gonads and adrenals as sources of 17-ketosteroids.

As has been noted earlier, the urine of subjects with adrenocortical hyperplasia or adrenocortical tumors contained large amounts of both 11-desoxy- and 11-oxy-17-ketosteroids.

In 1948 Mason (35) reviewed the reported measurements of the urinary excretion of steroids in adrenal disease. He noted that the urinary levels of 17-ketosteroids in cases of Addison's disease were low and that androsterone the only identifiable compound. In a limited number of subjects with adrenocortical tumors the urinary excretion of dehydroisoandrosterone was high in all but one instance, while in cases of adrenocortical hyperplasia the levels of this steroid were very low. Androsterone appeared to be elevated in subjects with adrenocortical hyperplasia. As early as 1939 it was suggested by Crooke and Callow (36) that it might be possible to distinguish between an adrenocortical tumour and adrenocortical hyperplasia on the basis of the total urinary 17-ketosteroid values; those having a tumour would have a very elevated level and those having hyperplasia would have a high normal or only a moderately elevated excretion. This idea is now known to be incorrect since the 17-keto-

steroid excretion can be normal in subjects with adrenocortical tumors (37, 38). Using more modern methods a number of workers have shown that in cases of adrenal disorder with elevated urinary 17-ketosteroids the relative amounts of the individual 17-ketosteroids vary considerably from subject to subject. In many of the earlier investigations the steroid conjugates were cleaved by boiling in the presence of strong acid. This is now known to destroy considerable quantities of the 17-ketosteroids present in urine.

Gemzell et al (39) reported in 1953 that following the removal of the adrenals from patients previously gonadectomized for prostatic carcinoma, the urinary 17-ketosteroid level decreased to zero. These findings indicated that the adrenal was the site of elaboration of these steroids.

The pituitary hormone, adrenocorticotropin (ACTH), which stimulates adrenal secretion increases the levels of urinary 17-ketosteroids when given to normal subjects (40, 41, 42). Landau et al (43) studied 17-ketosteroid excretion in normal adults following the administration of ACTH and obtained a large increase in the excretion of dehydroisoandresterone as well as of total 17-ketosteroids. Human chorionic gonadotrophin administration, which is known to stimulate the testes, leads to an increase in total 17-ketosteroid excretion but not in dehydroisoandrosterone excretion. Landau et al compared the quantities of 17-ketosteroids excreted following the administration of ACTH and of dehydroisoandrosterone. These authors concluded that dehydroiso-

androsterone may account for the major portion of the 17-ketosteroids of adrenal origin. Ronzoni (44) also obtained a large increase in urinary dehydroisoandrosterone following adrenal stimulation with ACTH. Kappas and Gallagher (28) found that ACTH increased the excretion of dehydroisoandrosterone more than those of the other individual 17-ketosteroids. The 11-oxy-17-ketosteroids as a group were elevated more than the 11-desoxy steroids. When the release of ACTH by the pituitary is suppressed by the administration of cortisone the levels of urinary dehydroisoandrosterone, androsterone and etiocholanolone decrease (45).

The studies mentioned above, as well as many others, indicate that the adrenal glands are the source of all the 11-oxy-17-ketosteroids and the major fraction of the 11-desoxy-17-ketosteroids. The presence of 11β-hydroxylase activity has never been demonstrated in normal gonadal tissue.

The testes secrete a precursor which gives rise to a small fraction of the androsterone and etiocholanolone since the urinary excretions of these two steroids are lower for castrated men than for normals (46). Castration leads to a large decrease in the urinary 17-ketosteroids in patients with prestatic cancer (47). Human chorionic gonadotrophin (HCG) administration, which does not stimulate the adrenals, causes an increase in urinary 17-ketosteroids and this respense is less evident in older than in young men. The plasma levels of 17-hydroxycorticosteroids and dehydroisoandrosterone are not significantly changed by HCG administration (48). However, the androsterone levels are significantly increased in males but not in females. These observations support the

view that dehydroisoandrosterone is principally of adrenal origin and the testes can elaborate precursors of androsterone and etiocholanolone.

Venning et al (49) reported very large amounts of urinary 17-ketosteroids in the urine of a subject with an interstitial cell tumor of the testes. They isolated androsterone sulphate in considerable yield from the urine of this patient.

It is believed that the normal ovary does not secrete significant quantities of 17-ketosteroid precursors but increased secretions may occur in pathological conditions such as ovarian tumors or polycystic ovaries as discussed by Dorfman (50). MacDonald et al (51) studied rates of secretion of dehydroisoandrosterone by normal female subjects with and without adrenal or ovarian suppression, by subjects with abnormal ovarian function, by a subject with Addison's disease and a castrate female. Their results indicated that the normal ovary secreted very small quantities of dehydroisoandrosterone and either Δ^4-androstenedione or testosterone. The normal ovary has the capacity to synthesize 17-ketosteroid precursors as will be discussed later in the section on biosynthesis.

STEROIDS PRESENT IN ENDOCRINE GLANDS AND THEIR VENOUS EFFLUENTS

A number of the 17-ketosteroids and their precursors have been isolated from the adrenals, testes and ovaries and the venous blood leaving these tissues.

1. Adrenals: A large number of steroids have been isolated from adrenal tissue and only those which are pertinent to this discussion will be described. In 1941 Pfiffner and North (52) reported the isola-

tion of 17α-hydroxyprogesterone from bovine adrenals. The C_{19} steroid, Δ^4-androstenedione, was isolated from hog adrenals by von Euw and Reichstein (53). Neher and Wettstein (54) reported the isolation of pregnenolone and 17α-hydroxypregnenolone from hog adrenals in 1960.

Dehydroisoandrosterone has been isolated from one human adrenal cortical tumor (55) and tentatively identified in another (56). Bloch et al (57) obtained evidence for the presence of Δ^4-androstenedione, dehydroisoandrosterone, 11β-hydroxyandrostenedione and hydrocortisone in human fetal adrenals.

In 1955, Pincus and Romanoff (58) identified hydrocortisone and 11β-hydroxyandrostenedione and tentatively identified Δ^4-androstenedione in adrenal vein blood from a man with metastatic prostatic carcinoma and a woman with metastatic breast carcinoma. In 1956 Bush et al (59) reported that they found hydrocortisone, 11β-hydroxyandrostenedione, dehydroisoandrosterone and androsterone in adrenal vein blood from a patient with virilism. Bush and Mahesh more recently (60) identified hydrocortisone, 11β-hydroxyandrostenedione and dehydroisoandrosterone, by means of paper chromatography, in adrenal venous blood from a patient with adrenocortical hyperfunction association with hirsutism. In both of these studies, Bush and his co-workers concluded that the concentration of dehydroisoandrosterone in the adrenal venous blood was too low to account for the total dehydroisoandrosterone production as estimated from the urinary level. It was suggested that there must be another source of at least a fraction of the excreted dehydroisoandrosterone.

Lombardo et al (61) analyzed 12 samples of human adrenal venous blood and found dehydroisoandrosterone in only one of them. These investigators found 17α-hydroxyprogesterone and 11-desoxyhydrocortisone in five and 11β-hydroxyandrostenedione in ten samples. Migeon (62) found 11β-hydroxyandrostenedione and Δ^4-androstenedione in adrenal vein blood from a patient with Cushing's syndrome due to bilateral adrenal hyperplasia. They also found Δ^4-androstenedione and etiocholanolone in the venous blood from a patient with an adrenal rest tumor. More recently, Hirschmann et al (63) have isolated dehydroisoandrosterone, Δ^4-androstenedione and 11β-hydroxyandrostenedione from the adrenal venous blood of normal female subjects. Trace quantities of Δ^5-androstene-3β,17β-diol were found. It was concluded that the levels of secretion of Δ^4-androstenedione and dehydroisoandrosterone were sufficient to account for the urinary 17-ketosteroids provided the observed secretion rates were maintained for 24 hours.

Dehydroisoandrosterone, Δ^4-androstenedione, 11β-hydroxyandrostenedione and 17α-hydroxyprogesterone have been isolated from adrenal venous blood obtained from three post menopausal women with mammary carcinoma who had received ACTH (64). Recently, Wieland et al (65) reported the finding of 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, dehydroisoandrosterone and Δ^4-androstenedione in adrenal venous blood from a patient with an adrenocortical tumor.

Of interest in the search for the precursors of the urinary 17-desoxy-17-ketosteroids are the very recent findings of conjugated steroids in adrenocortical tissue and adrenal venous blood. Baulieu

(66) analysed a large adrenal tumor and the venous blood coming from it and found free Δ^4-androstenedione and 11β-hydroxyandrostenedione in the tumor tissue but no free dehydroisoandrosterone. However, dehydroisoandrosterone sulphate was found in high concentrations in both the tumor and the adrenal venous plasma. The concentration of dehydroisoandrosterone sulphate was higher in the adrenal venous plasma than in the peripheral venous plasma. The concentrations of androsterone sulphate and etiocholanolone sulphate were higher in the peripheral venous blood. These results were confirmed in a study on a second patient. However, a small amount of free dehydroisoandrosterone was also found in the tumor. In the first subject studied 7-ketodehydroisoandrosterone sulphate was found in the adrenal venous blood and at a higher concentration than in the peripheral venous blood. This is the first report of this steroid in blood and suggests that this compound is formed in the adrenal gland rather than in peripheral tissue from dehydroisoandrosterone. Wieland et al (67) have recently reported the isolation of dehydroisoandrosterone sulphate from the adrenal vein blood of a normal subject at a concentration much higher than that of the free compound.

2. Testes: Little work has been done on the isolation of steroids from human testicular tissue. Testosterone was isolated from human testes in 1935 (68). A number of C_{19} steroids and possible precursors of the C_{19} steroids have been isolated from animal testes. Neher and Wettstein isolated dehydroisoandrosterone (54) from bovine testes and Ruzicka and Prelog (69) and Haines et al (70) have isolated pregnenolone, an intermediate in steroid biosynthesis, from hog testes. Dehydroiso-

androsterone has never been found in human testicular tissue or human spermatic venous blood.

Lucas et al (71) identified testosterone in a pool of spermatic blood collected from patients with prostatic carcinoma. Hollander and Hollander (72) were able to measure testosterone in human spermatic venous blood. Testosterone, Δ^4-androstenedione and 17α-hydroxyprogesterone have been isolated from bovine spermatic vein blood (73).

3. Ovaries: In 1950, Parkes (74) reported bioassay data which suggested that ovarian tissue can produce C_{19} steroids. Zander (75) in 1958 reported the isolation of androstenedione from extracts of human ovarian follicles and corpora lutea.

In this study, Zander also identified progesterone, and 17α-hydroxypregesterone, which is a known precursor of C_{19} steroids.

A number of steroids have been isolated from ovaries obtained from subjects with the Stein-Leventhal syndrome; this type of polycystic ovary is believed by many to produce significant quantities of androgens. Short (76) analysed cyst fluid from such ovaries and found measurable quantities of 17α-hydroxyprogesterone, Δ^4-androstenedione and dehydroisoandrosterone. Since no estrogens were detected a defect in hydroxylation at carbon-19, with a resulting accumulation of C_{19} steroids was suggested. Starka et al (77) isolated dehydroisoandrosterone and Δ^4-androstenedione from sclerocystic human ovaries with the former being present in higher concentrations. Mahesh and Greenblatt (78) isolated dehydroisoandrosterone or Δ^4-androstenedione and 17α-hydroxyprogesterone from the polycystic ovaries of patients with the Stein-Leventhal syndrome. When two patients with this syndrome were

given human follicular stimulating hormone (FSH) prior to ovariectomy, the ovaries contained 17α-hydroxypregnenolone as well as dehydroisoandrosterone. The latter steroid was present in concentrations higher than those found in the ovaries of subjects who did not receive FSH. Although testosterone has been isolated from arrhenoblastoma tissue on two occasions (79, 80), none was found in the studies of the normal or polycystic ovaries.

Studies on patients with polycystic ovaries showed that the levels of 17-ketosteroids in ovarian venous blood were higher than those in the peripheral blood (81). No attempts were made to isolate the individual compounds in this study. Mahesh et al (82) found small quantities of Δ^4-androstenedione in ovarian venous blood from normal subjects following FSH administration. Dehydroisoandrosterone and testosterone were not detected. No C_{19} steroids were found when the subjects had not received FSH. Recently, Mikhail et al (83) have reported the analysis of human ovarian venous blood at various intervals during the menstrual cycle. Progesterone levels decreased with time following ovulation and none was found in blood taken from an ovarian vein during an anovulatory cycle. In the samples in which progesterone was found, 17α-hydroxyprogesterone, Δ^4-pregnen-3-on-20α-ol and Δ^4-androstenedione were also found.

BIOSYNTHESIS OF C_{19} STEROIDS.

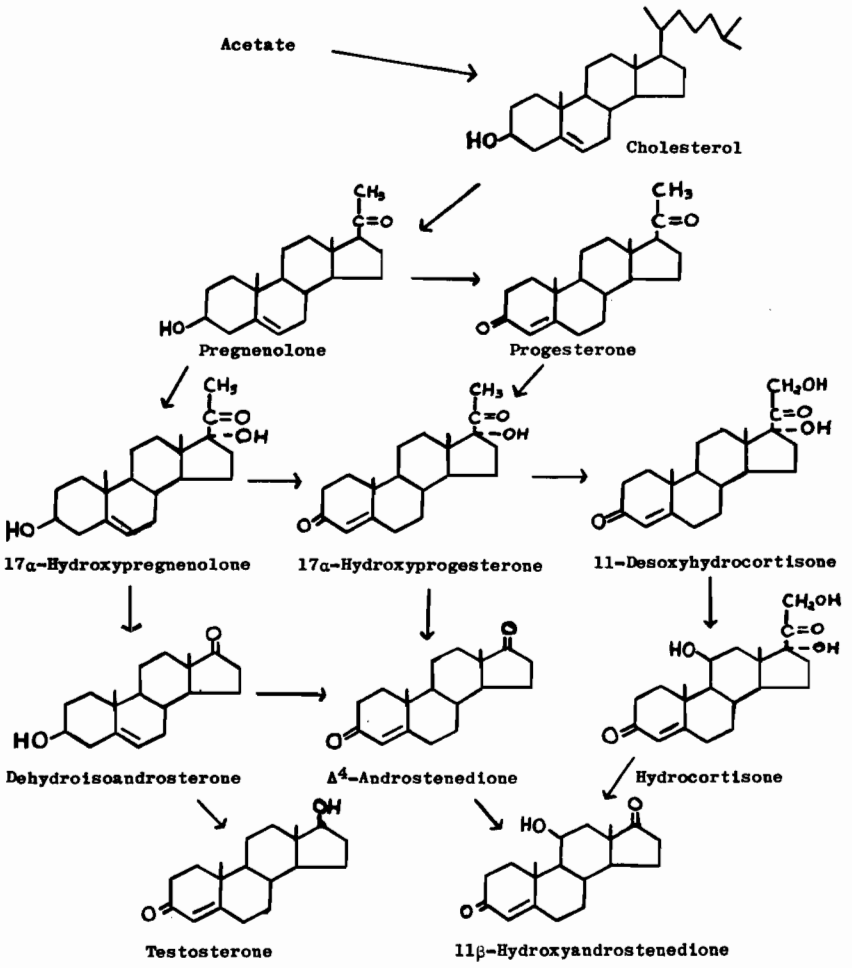
The pathways for the biosynthesis of C_{19} steroids in the adrenals and gonads has been studied using a number of in vitro techniques. These methods include perfusions of the whole gland and incubations with

tissue slices, whole homogenates and cell free systems. Present knowledge of biosynthetic pathways are summarized in Figure 2. In brief, the biosynthesis of the steroids involves the formation of cholesterol from acetate units and the degradation of the cholesterol to yield pregnenolone. The pregnenolone can either be converted to progesterone or hydroxylated at the 17- position to form 17α-hydroxypregnenolone. The intermediate, 17α-hydroxyprogesterone, can be formed by hydroxylation of progesterone or it can be formed from 17α-hydroxypregnenolone. Cleavage of the side chains of 17α-hydroxypregnenolone and 17α-hydroxyprogesterone yields dehydroisoandrosterone and Δ^4-androstenedione, respectively. Dehydroisoandrosterone can be converted to Δ^4-androstenedione. The Δ^4-androstenedione can be reduced at position 17 to form testosterone, or undergo 11-hydroxylation to yield 11β-hydroxyandrostenedione. The latter compound can also result from the cleavage of the side chain of hydrocortisone which is formed by hydroxylation of 17α-hydroxyprogesterone at first the 21 and then the 11 positions. It is believed that hydroxylations at positions 21 and 11 occur only in adrenocortical tissue under normal circumstances. The other reaction sequences are believed to be the same for the adrenals, testes and ovaries.

The formation of dehydroisoandrosterone is the subject of major importance in this study and so it will be discussed in detail. Most of the studies on the formation of dehydroisoandrosterone have been performed with adrenocortical tissue. Earlier studues by Bloch et al (84, 85) demonstrated the conversion of C^{14}-acetate to C^{14}-labelled dehydroisoandrosterone, Δ^4-androstenedione and 11β-hydroxyandrostene-

Figure 2.

PATHWAYS OF C_{19} STEROID BIOSYNTHESIS



dione by human adrenal slices obtained from a subject with adrenogenital syndrome. ACTH did not increase the yield of dehydroisoandrosterone although it enhanced the formation of 11β-hydroxyandrostenedione. An homogenate of an adrenal adenoma converted both C^{14}-cholesterol and tritium labelled pregnenolone to dehydroisoandrosterone (86). A metabolite which was not identified but was found to contain tritium and no carbon-14 led to the suggestion that cholesterol might be going to dehydroisoandrosterone without passing through pregnenolone but this pathway has not been confirmed. Goldstein et al (87) demonstrated the conversion of pregnenolone to dehydroisoandrosterone and 11β-hydroxyandrostenedione by an adrenal adenoma from a patient with Cushing's syndrome.

Evidence that adrenal tissue can form the proposed intermediate between pregnenolone and dehydroisoandrosterone, namely, 17α-hydroxypregnenolone, has come from the work of Weliky and Engel (88) who studied the metabolism of pregnenolone-H^3 and progesterone-C^{14} by hyperplastic adrenal gland slices. Its formation was suggested by the distribution of radioactivity in the partition column chromatogram and the counter current distribution fractionation. Unfortunately, the 17α-hydroxypregnenolone was not further characterized. Both labels were present in 17α-hydroxyprogesterone and hydrocortisone but there was no tritium in the column eluates containing progesterone, suggesting that pregnenolone was hydroxylated to form 17α-hydroxypregnenolone and this was converted to 17α-hydroxyprogesterone. This latter conversion has been shown for normal adrenal gland slices (89). The conversion

of 17α-hydroxypregnenolone to dehydroisoandrosterone has been reported in very small yields for bovine adrenal homogenates (90) and human adrenal slices (89).

Cohn and Milrow (91) incubated slices of normal, atrophic 'hypertensive', hyperplastic, adenomatous and carcinomatous adrenals of human origin for three hours in Krebs-Ringer bicarbonate buffer. The authors found that dehydroisoandrosterone, Δ^4-androstenedione and 11β-hydroxyandrostenedione were released into the incubation medium. The release of 11β-hydroxyandrostenedione but not of the other two compounds was stimulated by ACTH. Conversions of labelled 17α-hydroxypregnenolone to dehydroisoandrosterone in 1.6 and 1.4 per cent yield to Δ^4-androstenedione in 6.8 and 5.6 per cent yield, and to 11β-hydroxyandrostenedione in 7.2 and 6.8 per cent yield were obtained with adenomatous and hyperplastic adrenal slices, respectively. The conversions of 17α-hydroxypregnenolone to Δ^4-androstenedione and 11β-hydroxyandrostenedione were greater than the conversion of progesterone to the same compounds, suggesting that they were formed mainly by was of dehydroisoandrosterone and not by way of 17α-hydroxyprogesterone. In agreement with the findings of conjugated steroids in adrenals and adrenal vein blood, several workers have demonstrated the in vitro formation of dehydroisoandrosterone sulphate by adrenal tissue. The formation of dehydroisoandrosterone sulphate from the free compound has been shown using homogenates of normal (92), carcinomatous (93) and adenomatous (94) adrenal tissue. In this last report, Cohn et al, also demonstrated the conversion of 17α-hydroxypregnenolone to dehydroisoandrosterone sulphate. In a recent note, Lebeau and Baulieu (95)

have reported that the supernatant obtained by centrifugation of an adrenal tumor homogenate sulphated dehydroisoandrosterone to the extent of 98 per cent and other steroids to a lesser degree.

There have been fewer demonstrations of dehydroisoandrosterone synthesis by gonadal tissue. Conversions of labelled pregnenolone to 17α-hydroxypregnenolone and dehydroisoandrosterone have been shown to be effected by minces of human fetal testes (96). Bovine testes have been shown to convert 17α-hydroxypregnenolone to dehydroisoandrosterone (90). Ryan and Smith (97) incubated C^{14}-acetate with minces of follicular cyst linings of normal ovaries and positively identified dehydroisoandrosterone and pregnenolone as incubation products and obtained some evidence for the formation of 17α-hydroxypregnenolone. Ovarian tissue from a subject with the Stein Levanthal syndrome converted pregnenolone to dehydroisoandrosterone (98).

PERIPHERAL FORMATION OF URINARY 17-KETOSTEROIDS IN MAN

There are many reports in the literature of the in vivo and in vitro metabolism of the steroids which are known to be secreted by the endocrine tissues and which are converted to the 17-ketosteroids. The in vivo transformations of the adrenal and gonadal C_{21} and C_{19} compounds to 17-ketosteroids will be discussed briefly in this section. Present knowledge of the conjugation of the 17-ketosteroids will also be discussed briefly.

THE CONVERSION OF C_{21} STEROIDS TO 17-KETOSTEROIDS

There have been a number of reports on the in vivo metabolism of hydrocortisone, the major glucocorticoid secreted by the adrenal

cortex. The interest in this and the other 11-oxygenated compounds lies in the possibility that they may be precursors of the urinary 11—oxy-17-ketosteroids. In 1953, Burstein et al (99) reported on the urinary steroids found after the oral administration of 2.5 gm. of hydrocortisone acetate to a man with scleroderma. Besides the C_{21} metabolites, 11β-hydroxyetiocholanolone, 11-ketoetiocholanolone and 11β-hydroxyandrosterone were isolated in quantities greater than those found in the control urines. The actual per cent conversions were very small. Cortisone acetate, when given orally to male subjects, was also shown to be converted in low yields to 11β-hydroxyetiocholanolone, 11-ketoetiocholanolone and 11β-hydroxyandrosterone (100, 101).

Sandberg et al (102) obtained preliminary evidence for the conversion of C^{14}-hydrocortisone to urinary 11-ketoetiocholanolone and 11β-hydroxyetiocholanolone. Fukushima et al (103) studied the metabolism of a tracer dose of C^{14}-hydrocortisone in normal men and found conversions to urinary 11-ketoetiocholanolone plus 11β-hydroxyetiocholanolone ranging from 2 to 12 per cent. Very little labelled 11β-hydroxyandrosterone was found in the urine.

Bradlow et al (104) administered labelled hydrocortisone which had been reduced at the C-20 position and found that the distribution of the label in the pattern of urinary metabolites was similar to that following the administration of hydrocortisone. A total of 4.5 per cent of the radioactivity in the neutral extract was identified as 11-ketoetiocholanolone and 11β-hydroxyetiocholanolone. They concluded that reduction of the ketone at C-20 was not a major first step in the

metabolism of hydrocortisone and that it had no qualitative effect on the cleavage of the side chain to form 17-ketosteroids.

In 1951, Fajans et al (105) reported that the oral administration of 11-desoxyhydrocortisone (Compound S) led to an elevation in the urinary 17-ketosteroid excretion. Birke (106) and Birke and Plantin (107) identified etiocholanolone and androsterone as being quantitatively only very minor metabolites of 11-desoxyhydrocortisone. The compound under normal conditions probably contributes very little to the urinary 17-ketosteroids because of its low level of secretion which is evident from the low excretion of its principal urinary metabolite, tetrahydro-11-desoxyhydrocortisone (108).

A number of investigations to study the in vivo conversion of 21-desoxysteroids to 17-ketosteroids have been reported. These compounds are secreted in large quantities by patients with adrenal hyperplasia due to defects in the hydroxylation of the steroid molecule and it was suggested that these compounds could be the source of the large quantities of 17-ketosteroids excreted by these subjects. It was also possible that these 21-desoxy steroids might be quantitatively significant as precursors of the 17—ketosteroids excreted under normal conditions. Burstein et al (109) obtained only very small conversions of orally administered 21-desoxycortisone to the 11-oxy-17-ketosteroids. Jailer et al (110) isolated both the 11-keto and 11β-hydroxy etiocholanolones and the 11-keto and 11β-hydroxy androsterones following the oral administration of 21-desoxycortisone. Rosselet et al (111), using large quantities of orally administered 21-desoxycortisone, and Fukushima et

al (112), using tracer doses of the labelled compound, obtained low conversions to urinary 11-ketoetiocholanolone, 11-ketoandrosterone, 11β-hydroxyetiocholanolone and 11β-hydroxyandrosterone. A number of studies concerned with the metabolism of 17α-hydroxyprogesterone in humans have been reported. Jailer et al (110) administered orally large quantities of 17α-hydroxyprogesterone and obtained small increases in the urinary etiocholanolone and androsterone fractions. The ratio of the increase in the etiocholanolone fraction to that in the androsterone fraction was 5:1. It could be concluded from this result that 17α-hydroxyprogesterone secretion would have to be increased enormously to be quantitatively important as a precursor of androsterone. Similar results were obtained by others (113, 114). Vermeulen et al (115) obtained less than a 0.5 per cent conversion of a trace dose of 17α-hydroxyprogesterone-C^{14} to labelled etiocholanolone. Fukushima and his co-workers (116, 117) have studied the metabolism of C^{14}-labelled 17α-hydroxyprogesterone in a normal subject and in several subjects with adrenal carcinoma. They were able to isolate labelled androsterone and etiocholanolone in all cases. The yields of thetwo were small and there was always more radioactivity in the etiocholanolone fraction.

Because the 3β-hydroxy-Δ^5 structure is present in both dehydroisoandrosterone and 17α-hydroxypregnenolone, there have been several recent studies on the metabolism of this C_{21} steroid to urinary 17-ketosteroids. Intravenous infusion of 17α-hydroxypregnenolone to an adrenalectomized dog led to an increase in the plasma level of dehydroisoandrosterone (118). Solomon et al (119) obtained a 4.3 per cent conversion of labelled 17α-hydroxypregnenolone to urinary dehydroisoandrosterone, etiocholanolone and androsterone in a patient with

adrenocortical carcinoma, and a conversion of less than 0.1 per cent in a normal subject. Roberts et al (120) obtained a 3 per cent conversion in a similar study in a person with an adrenal adenoma. The conversion of 17α-hydroxypregnenolone to urinary 17-ketosteroids will be discussed in greater detail later.

IN VIVO METABOLISM OF C_{19} STEROIDS TO THE 17-KETOSTEROIDS FOUND IN URINE.

The metabolism of testosterone has been the subject of numerous studies in man. In 1939, Callow (121) studied the metabolism of orally administered testosterone and isolated androsterone and etiocholanolone as minor metabolites. Dorfman et al (122) isolated androsterone in the urine following the oral and intramuscular administration of testosterone. In 1940, Dorfman and Hamilton (123, 124) reported that the oral administration of testosterone, androsterone, dehydroisoandrosterone, androstane-3α,17β-diol, androstane-3,17-dione or Δ^4-androstenedione all led to increases in the level of the urinary 17-ketosteroids. The level of androsterone in urine was shown to be increased after the administration of each of the above compounds with the exception of dehydroisoandrosterone. Of the administered androsterone itself, 24 per cent was excreted in the urine unchanged. Androstanediol, Δ^4-androstenedione and androstanedione were suggested as intermediates in testosterone metabolism. Sheller et al (125) administered orally a large quantity of testosterone to a normal female and isolated androsterone and etiocholanolone in yields of 14.6 and 7.7 per cent respectively.

Other C_{19} compounds of the etiocholane and androstane series which are metabolised in vivo by man to urinary 17-ketosteroids are androstan-17β-ol-3-one, which is converted to isoandrosterone and androsterone, and etiocholan-17β-ol-3-one, which is converted to etiocholan—3α,17β-diol, and etiocholanolone (126). In 1950 Dorfman et al (127) reported on the metabolism of Δ^4-androstenedione to androsterone and etiocholanolone and of androstanedione and androstanediol to androsterone and isoandrosterone. Orally administered isoandrosterone can be converted to urinary androsterone (128, 129).

Fukushima, Gallagher and their co-workers used deuterium labelled testosterone to study its metabolism in vivo (130, 131, 132) and found androsterone and etiocholanolone to be the major metabolites formed. Minor quantities of etiocholane-3α,17β-diol, etiocholane-3,17-dione, androstane—3α,17β-diol and isoandrosterone also were excreted. When deuterium labelled Δ^4-androstenedione was administered to a male subject 43 per cent of the dose was excreted within 24 hours as androsterone and etiocholanolone.

Following the intravenous administration of C^{14}-testosterone almost all the radioactivity is excreted within 48 hours with over 50 per cent appearing in the urine during the first 4 hours (133). Thus it appears that testosterone is metabolized and cleared from the body very rapidly. Between 12 to 14 per cent of the radioactivity appeared in the bile of subjects with T-tube drainage. Only 6 per cent was excreted in the stools of normal subjects. In a second study (134) blood was taken from the subjects at intervals of 15 to 240 minutes

following the injection of C^{14} testosterone. Labelled etiocholanolone, androsterone, isoandrosterone and testosterone were identified in the plasma extract and there was chromatographic evidence of labelled androstane-3,17-dione and etiocholane-3α,17β-diol. This study further indicated that testosterone is transformed rapidly to its metabolites.

The studies just described indicate that testosterone is converted by man to androsterone and etiocholanolone. However, present knowledge indicates that dehydroisoandrosterone is quantitatively the most important precursor of these two urinary 17-ketosteroids. Urinary dehydroisoandrosterone, androsterone, etiocholanolone and Δ^5-androstene—3β,17β-diol accounted for 28 per cent of the dehydroisoandrosterone administered intramuscularly to a man with anterior pituitary insufficiency (135). Isoandrosterone was not found. In a similar study (136) in two subjects with Addison's disease, Δ^5-androstene-3β,16α,17β-triol was isolated as a metabolite but no dehydroisoandrosterone was detected. Etiocholane—3α,17β-diol was identified in the urine of a subject receiving a large dose of dehydroisoandrosterone although the yields of androsterone and etiocholanolone were considerably less than those found in the two studies previously mentioned (137). Ungar et al (138) reported the in vivo metabolism of orally administered Δ^5-androstene-3β,17βdiol to urinary dehydroisoandrosterone, androsterone, etiocholanolone and etiocholane-3α,17β-diol by a human subject.

Kirschner et al (139) studied the metabolism of large amounts of orally administered dehydroisoandrosterone at different dose levels in normal subjects and found that although the level of dehydroisoandrosterone in the urine was highest with the largest dose, the

fraction of the administered compound excreted unchanged was lowest with the largest dose. Etiocholanolone was excreted in proportionately larger quantities with the large dose than was androsterone. The total percentage of the administered dose excreted as dehydroisoandrosterone, androsterone and etiocholanolone was lowest for the highest dose.

Lieberman and his co-workers (26, 140 - 144) have carried out a number of studies on the invivo metabolism of dehydroisoandrosterone using tracer doses of the labelled compounds and have made a number of interesting findings. They have measured the specific activity of the urinary dehydroisoandrosterone and used this figure to calculate the secretion or production rate of dehydroisoandrosterone. Their results in normal subjects ranged from 15 to 25 mg per 24 hours. In their first studies, they found that the specific activities of the androsterone and etiocholanolone were the same as that of the dehydroisoandrosterone. These results indicated that dehydroisoandrosterone was the principal precursor of the 11-desoxy-17-ketosteroids, i.e. the etiocholanolone and androsterone were not being diluted by other precursors such as testosterone to any significant degree. In subsequent studies they found that in a number of cases the specific activities of the androsterone and etiocholanolone were significantly higher than that of the dehydroisoandrosterone which had been excreted as the sulphate. Attempts to explain this finding prompted further studies. They found no significant daily variation in the secretion of dehydroisoandrosterone. This is in contrast to the earlier results of Pincus et al (145) who had reported a diurnal variation in the excretion of 17-ketosteroids,

and Migeon et al (146) who reported a diurnal variation in plasma dehydroisoandrosterone and androsterone. Lieberman and his co-workers also studied the influence of the level of secretion of dehydroisoandrosterone on its metabolism and found that when the secretion rate was altered in a normal subject the fraction of the tracer dose of dehydroisoandrosterone excreted unchanged varied in proportion to the level of secretion while the fraction excreted as androsterone plus etiocholanolone did not vary. Other aspects of these studies will be discussed in a later section. It is of interest that Lipsett (147) in studies of the metabolism of labelled dehydroisoandrosterone found that the specific activities of the urinary dehydroisoandrosterone, androsterone and etiocholanolone were the same for two ovariectomized patients but the specific activities of androsterone and etiocholanolone were lower than that of dehydroisoandrosterone for three patients with adrenal carcinoma, indicating that there were precursors other than dehydroisoandrosterone for these two compounds in the cancer patients.

Androsterone and etiocholanolone can also be converted to other steroids by man. The results of several investigations suggest that androsterone and etiocholanolone are probably more important quantitatively as metabolites of testosterone and dehydroisoandrosterone than is indicated by the urinary levels of the two 17-ketosteroids. Gallagher et al (132) studied the metabolism of deuterium labelled androsterone and etiocholanolone administered intramuscularly to humans. Approximately 25 per cent of the androsterone and 50 per cent of the etiocholanolone administered were excreted unchanged within 24 hours. Kappas et al (148) measured the levels of urinary metabolites following

the intramuscular administration of a large amount of etiocholanolone. From 75 to 90 per cent of the administered steroid was excreted unchanged. Insignificant quantities were excreted as etiocholane-3,17-dione or etiocholan-3α,17β-diol. When etiocholane-3β-ol-17-one was given, the principal metabolite was etiocholanolone (12.9 per cent of dose) while only 0.6 per cent was excreted unchanged.

Slaunwhite and Sandberg (149) studied the metabolism of C^{14}-etiocholanolone and C^{14}-androsterone. The radioactivity was excreted in the urine quite rapidly (50 to 75 per cent in 4 hours) and the average recoveries of radioactivity during the 48 hours following the administration were 97 per cent for etiocholanolone and 83 per cent for androsterone. Only 55 to 65 per cent of the injected radioactivity was recovered unchanged in the urine and they concluded that a larger portion of administered testosterone may be converted to androsterone and etiocholanolone than is indicated by the isolation of these metabolites from urine.

Fukushima and his co-workers (150) administered etiocholanolone labelled with tritium in the 3β-position and found that only 55 per cent of the administered radioactivity appeared in the urine as etiocholanolone. Most of the radioactivity not found in the etiocholanolone was present in the urine in the form of tritium labelled water and other non-steroidal compounds. This indicated that there is considerable oxidation of the 3α-hydroxyl group after its formation.

It was previously mentioned that the administration of etiocholanolone and androsterone in large quantities led to the isolation

of 18-hydroxyetiocholanolone and 18-hydroxyandrosterone, respectively, from urine (23). These findings also indicate that androsterone and etiocholanolone are not end products of metabolic sequences. Scheider and Lewbart (21) also found 16α-hydroxyandrosterone after the administration of androsterone. Several non-ketonic steroids which are probably metabolites of androsterone and etiocholanolone have been isolated from urine. These compounds are etiocholan-3α,17β-diol, androstan-3α, 17β-diol, etiocholan-3α,16α,17β-triol and androstan-3α,16α,17β-triol (151).

Bradlow et al (152) studied the metabolism of dehydroisoandrosterone labelled in the 3α-position with tritium to determine whether oxidation of the 3 - hydroxyl group occurred during the formation of ispandrosterone or whether it was formed by reduction of the double bond without prior oxidation. The isolated isoandrosterone had no tritium in the 3α position indicating that dehydroisoandrosterone was metabolized via a 3-ketone intermediate. Only a small frantion of the administered dehydroisoandrosterone was excreted unchanged (3.4 and 2.7 per cent of dose). There was a small amount of tritium in both the urinary androsterone and isoandrosterone but not at position 3, suggesting that some of the tritium removed during oxidation of the 3-hydroxyl group was used in the reduction of the double bond.

The oxidation of the 3β-hydroxyl group on dehydroisoandrosterone is irreversible as the administration of 3-keto-Δ^5-androsten-17—one does not yield dehydroisoandrosterone (150).

From an examination of the urinary metabolites excreted in studies using C_{19} and C_{21} steroidal precursors, Dorfman (153, 154) advanced a

general rule which may serve as a guide in predicting the ratio of 5α-reduced metabolites to 5β-reduced metabolites in the urine. C_{19} steroids containing a 3β-hydroxy-Δ^5 group or a Δ^4-3-ketone group plus a 17-ketone group give rise to urinary metabolites having a 5α/5β ratio of approximately 1. C_{19} steroids with a 17-ketone and a Δ^4-3-ketone plus an additional oxygen function at carbon 11, give rise to metabolites having a 5α/5β ratio much greater than one. C_{21} steroids with a Δ^4-3-ketone group lead to 17-ketosteroid metabolites with the 5β-configuration predominating. A comparison of the 5α/5β ratios of the urinary 17-ketosteroid metabolites arising from the C_{21} and C_{19} steroids has led to the conclusion that C_{21} steroids are transformed to 17-ketosteroids following reduction in ring A. If side chain cleavage occurred first, the product would be Δ^4-androstenedione which gives rise to urinary metabolites with a 5α/5β ratio of approximately one.

Although it is generally assumed that the liver is the organ principally responsible for the peripheral metabolism of steroids, there have been very few in vitro studies on the metabolism of dehydroisoandrosterone by liver tissue. In 1948, Schneider and Mason (155) reported that rabbit liver slices could convert dehydroisoandrosterone to Δ^5-androstene-3β,17β-diol and Δ^5-androstene-3β,16α,17β-triol. The sulphates of dehydroisoandrosterone and Δ^5-androstene-3β,17β-diol were also formed. A rabbit liver homogenate converted dehydroisoandrosterone to Δ^5-androstene-3β,17β-diol. Klempien et al (156) perfused dehydroisoandrosterone through an isolated dog liver and found evidence for two types of metabolism. The dehydroisoandrosterone was converted to

Δ^5-androstene-3β,17β-diol, testosterone and Δ^4-androstenedione. Dehydroisoandrosterone sulphate was also formed. There was no evidence for the formation of glucosiduronidates.

CONJUGATION OF 17-KETOSTEROIDS.

The fact that 17-ketosteroids are excreted as conjugates was recognized early in the study of steroid metabolism. Butenandt (1, 2) found that when he boiled urine with hydrochloric acid before extraction with chloroform he obtained an increased yield of androgenic material.

The first actual isolation of a 17-ketosteroid conjugate was that of androsterone sulphate from the urine of a man with an interstitial cell tumor of the testes. This finding by Venning et al (49) in 1942 was followed in 1944 by the report of Munson et al (157) on the isolation of dehydroisoandrosterone sulphate following the ingestion of the free compound.

An indication that androsterone and etiocholanolone are excreted as glucosiduronidates was obtained by Mason (35) who found that these steroids were released by a preparation of β-glucuronidase from rat liver. Following the intravenous administration of testosterone, West et al (158) isolated the steroid conjugate fraction of urine and after purification obtained good evidence for the presence of androsterone and etiocholanolone glucosiduronidates although they were unable to separate them.

Schneider and Lewbart (21) isolated and separated the urinary steroid conjugates following the administration of large quantities of

androsterone and dehydroisoandresterone to humans. The major urinary conjugate found after the androsterone had been given was androsterone glucosiduronidate. The major metabolite of the androsterone was isoandrosterone sulphate. Androsterone sulphate was also found in smaller quantities. The fraction of the steroids excreted as glucosiduronidates remained quite constant despite variation in the dosages. After giving dehydroisoandrosterone, large quantities of dehydroisoandrosterone sulphate were excreted. In addition, considerable quantities of androsterone and etiocholanolone glucosiduronidates were found and a small quantity of dehydroisoandrosterone glucosiduronidate was also isolated.

Dehydroisoandrosterone glucosiduronidate has recently been identified in normal human urine following the administration of a trace dose of labelled dehydroisoandrosterone (159).

It was previously thought that the formation of steroid conjugates was a mechanism for facilitating renal clearance, and that these conjugates were metabolic end-products. This concept was disproved by Roberts et al (160) with respect to the sulphates by administration of 7α-H^3-dehydroisoandrosterone sulphate to normal subjects and isolation from the urine of labelled androsterone and etiocholanolone which were excreted as glucosiduronidates. This indicated that dehydroisoandrosterone and its sulphate are interconvertible in the body.

The demonstration by Baulieu et al (161) that androstene-3β,17β-diol-3 sulphate can be converted to dehydroisoandrosterone sulphate without cleavage of the sulphate group provided evidence that a steroid sulphate can be further metabolized.

Siiteri and Lieberman (162) administered intravenously randomly tritiated androsterone glucosiduronidate to normal subjects. All the injected radioactivity was recovered in the urine as unchanged androsterone glucosiduronidate. This indicated that the glucosiduronidate is a metabolic end product.

The 17-ketosteroids in plasma exist mainly as conjugates. The first 17-ketosteroid isolated from plasma was dehydroisoandrosterone which was obtained only after acid hydrolysis (163). Androsterone was isolated from plasma by the same methods (164). Oertel and Eik Nes (165) reported the isolation of etiocholanolone and 11β-hydroxy-etiocholanolone from plasma following solvolysis and acid hydrolysis although the infra red spectra of their isolated compounds were not well defined. Baulieu (166) identified dehydroisoandrosterone, androsterone and etiocholanolone sulphates in plasma pools from 3 normal subjects and from 3 subjects receiving ACTH. Oertel and Eik Nes (167, 168) have reported the existence of complexes of dehydroisoandrosterone and phosphoric acid in human plasma but these results have not been confirmed.

A number of investigations have shown that the rate of clearance of a 17-ketosteroid from the body is dependent upon the nature of its conjugation. Bongiovanni and Eberlein (169) gave large doses of dehydroisoandrosterone ar androsterone to human subjects either orally or intravenously and found that androsterone was cleared by the kidney much faster than dehydroisoandrosterone. Since most of the dehydroisoandrosterone was excreted as the sulphate and most of the androsterone

as the glucosiduronidate, it was concluded that sulphates are not treated in the same way as glucosiduronidates by the kidney and hence are excreted more slowly. This could explain the higher blood level of dehydroisoandrosterone. The dehydroisoandrosterone is present as the sulphate in the blood.

Kellie and Smith (170) obtained results similar to that of Bongiovanni and Eberlein. They found that etiocholanolone glucosiduronidate was cleared as rapidly as androsterone glucosiduronidate. In one of their plasma fractions, they obtained evidence for the presence of the glucosiduronidates of dehydroisoandrosterone, 11-ketoetiocholanolone and 11β-hydroxyetiocholanolone.

The levels of unconjugated dehydroisoandrosterone, androsterone and etiocholanolone in the plasma under normal conditions are very low (171) and consequently a very sensitive method is necessary for their measurement. The levels of unconjugated dehydroisoandrosterone and etiocholanolone in the plasma are high in some patients with the so-called etiocholanolone fever. It was suggested that this may be due to a defect in the conjugation reactions. Cara et al (172) found a high level of plasma conjugated etiocholanolone in a patient with virilizing adrenal hyperplasia and periodic fever.

MacDonald et al (173) studied the excretion of 17-ketosteroid conjugates following the intravenous administration of labelled dehydroisoandrosterone. There were marked differences in the rate of appearance of the sulphates of dehydroisoandrosterone, androsterone and etiocholanolone. The excretion of etiocholanolone sulphate, etiocho-

lanolone glucosiduronidate and androsterone glucosiduronidate was almost complete within one day. It required 3 days for the excretion of 93 per cent of the dehydroisoandrosterone sulphate and 82 per cent of the androsterone sulphate.

There have been several in vitro demonstrations of steroid glucosiduronidate and sulphate formation. Cohn and Hume (174) perfused labelled androsterone and etiocholanolone through dog kidneys by means of cannulae in the renal artery and vein and obtained good evidence for the formation of androsterone and etiocholanolone glucosiduronidate by the kidney. When the two compounds were perfused simultaneously there was a higher yield of etiocholanolone glucosiduronidate than of androsterone glucosiduronidate and the suggestion was made that the equitorial hydroxyl of etiocholanolone was conjugated more rapidly than the axial hydroxyl of androsterone. This finding agrees with that of the more rapid clearance of etiocholanolone in humans as reported by others.

The in vitro formation of steroid sulphates has been the subject of a number of studies. De Meio et al (175) obtained some evidence for the formation of dehydroisoandrosterone sulphate by rat liver homogenates but they did not identify their products. Roy (176) studied the synthesis of dehydroisoandrosterone sulphate by a similar preparation and found the substrate specificity to be very low. Schneider and Lewbart (177) incubated 32 steroids with a microsome--free supernatant of a rabbit liver homogenate and obtained evidence that 14 of them were conjugated with sulphuric acid. These were mainly 3β-

hydroxy-Δ^5 steroids and steroids with a 5α-hydrogen. These findings are supported by the types of conjugates found in human urine, i.e. dehydroisoandrosterone, androsterone and isoandrosterone are excreted largely as sulphates while etiocholanolone (5β-hydrogen compound) is usually excreted as a glucosiduronidate. Testosterone was also conjugated with sulphuric acid in this system. Lipman (178) and Nose and Lipman (179) were able to separate by means of electrophoresis the enzyme fraction of rat liver responsible for conjugating 3β-hydroxy steroids from those responsible for conjugating estrogenic steroids and a general phenol conjugating fraction.

A general scheme indicating the pathways involved in the metabolism of C_{19} steroids according to present knowledge, is shown in Figure 3.

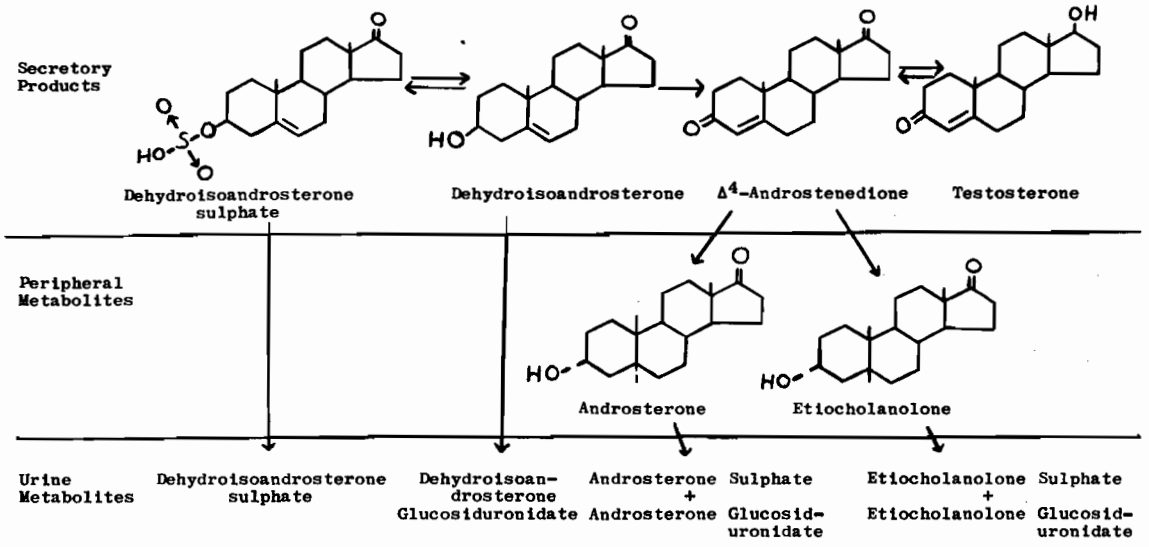
THE ORIGIN OF URINARY DEHYDROISOANDROSTERONE IN MAN

In 1938, Marker (180) discussed the origin and interrelationships of the steroidal hormones and suggested that dehydroisoandrosterone was a metabolite of Δ^4-androstenedione. He did not agree with previous postulates that dehydroisoandrosterone was derived from cholesterol via pregnenolone.

Even as late as 1952, Munson and Forsham (181) reported that dehydroisoandrosterone was present in the urine following the administration of cortisone and hydrocortisone to three men who were adrenalectomized and gonadectomized. It can thus be seen that there was still very much confusion regarding the origin of this 17—ketosteroid. The above two papers suggest the transformation of a Δ^4-3-ketone to a 3β-

Figure 3.

PERIPHERAL METABOLISM OF C_{19} STEROIDS



hydroxy-Δ^5 configuration. This conversion has never been shown to occur in the human.

In a series of papers (182 - 186), the Hirschmanns reported the isolations of a number of metabolites with the 3β-hydroxy-Δ^5 configuration from the urine of a boy with adrenal carcinoma. The quantities of dehydroisoandrosterone in the urine of this subject were very high. Other compounds isolated were Δ^5-androstene-3β,16α,17β-triol, Δ^5-pregnene-3β,17α,20α-triol, Δ^5-pregnene-3β,16α,20α-triol and Δ^5-pregnene-3β,20α-diol. The authors suggested that the precursor of the urinary Δ^5-pregnene-3β,20α-diol might be pregnenolone. It had been suggested earlier by other investigators that pregnanolone is an important intermediate in the biosynthesis of steroids. The Hirschmanns suggested that the isolations of considerable quantities of the Δ^5-pregnene-3β,20α-diol from urine indicated an excessive production of pregnenolone and this would account for the elevated excretion of the other steroids.

Lieberman and Teich (187) discussed in an editorial the possible precursors of dehydroisoandrosterone. The authors suggested that this precursor would have a 3β-hydroxy-Δ^5 configuration.

Steroids with the 3β-hydroxy-Δ^5 structure are present in considerable quantities in the urine of many subjects with adrenocortical carcinoma (22, 37, 38). With the exception of dehydroisoandrosterone, the levels of these compounds in normal urine are very low. Some of these 3β-hydroxy-Δ^5 steroids are now isolated regularly from normal urine as a result of the use of more improved methods.

In 1958, Fotherby (188) reported the isolation of three such 3β-hydroxy-Δ^5 steroids from normal urine for the first time. These compounds were Δ^5-androstene-3β,17β-diol, Δ^5-pregnene-3β,17α,20α-triol and Δ^5-androstene-3β,16β,17β-triol. This was the first isolation of the latter compound from a natural source. It was suggested in this report that the Δ^5-pregnenetriol might be a precursor of urinary dehydroisoandrosterone because it possessed the 17α-hydroxyl group which is necessary for side-chain cleavage.

Evidence for the formation of pregnenolone by the normal human was its isolation (189) from a plasma pool obtained from normal male subjects. The isolation of 17α-hydroxyprogesterone from normal human plasma was also reported in this study. Although the chromatographic evidence for these two compounds was good, the infra red spectra were poor. Oertel (190) isolated 17α-hydroxypregnenolone from the plasma of normal males and identified it by chromatography and infra red analysis. It was present at a concentration of 9.9 μg per liter of plasma.

In 1961, Wilson (191) analysed a number of urines for 3β-hydroxy-Δ^5 steroids and found Δ^5-pregnene-3β,17α,20α-triol in all specimens analysed. This was evidence for the secretion of the presumed precursor, 17α-hydroxypregnenolone. There was an inverse relationship between the levels of Δ^5-pregnenetriol and dehydroisoandrosterone, indicating that the level of the triol may be higher when there is a decreased conversion of 17α-hydroxypregnenolone to the 17-ketosteroid. The urinary steroid, Δ^5-pregnene-3β,20α-diol was also found in all the

urines examined. In a subject with adrenal carcinoma, amphenone caused a parallel decrease in both urinary pregnenetriol and dehydroisoandrosterone, thus indicating a common precursor. When a large dose of cholesterol-C^{14} was given to a patient with adrenal carcinoma, the specific activities of the urinary Δ^5-pregnenetriol and dehydroisoandrosterone was quite similar.

Cholesterol and pregnenolone are not quantitatively important as precursors of dehydroisoandrosterone. Burstein and Dorfman (192) reported very low conversions of these two steroids as well as mevalonate to urinary 17-ketosteroids by a female subject with an adrenal adenoma and a dehydroisoandrosterone excretion of 750 mg per day. A female subject with adrenal carcinoma also gave very small conversions of cholesterol to urinary 17-ketosteroids (24).

Ungar et al (193) studied the conversion of acetate-C^{14} to urinary steroids in two patients in the terminal stages of adrenal carcinoma. The authors were able to detect traces of radioactive dehydroisoandrosterone, androsterone, etiocholanolone, Δ^5-androstenediol and Δ^5-pregnenediol in the urine from one of the two patients.

In 1960, Solomon et al (119) reported the in vivo conversion of 17α-hydroxypregnenolone to dehydroisoandrosterone, androsterone and etiocholanolone by a patient with adrenal carcinoma and extensive metastases. The per cent conversion to these three compounds was 4.3 of which 65 per cent was accounted for by dehydroisoandrosterone. This patient was excreting large quantities of 17-ketosteroids (158 mg of dehydroisoandresterone, 18 mg of androsterone and 26 mg of etiocholanolone per 24 hours). The same experiment was repeated in a normal male

and the conversion was less than 0.1 per cent. Two possible explanations for this large difference were suggested. The first, the more plausible of the two, was that the large mass of metastatic tissue was responsible for the enhanced conversion; the second possibility was that the high levels of circulating dehydroisoandrosterone was affecting the conversion of the administered 17α-hydroxypregnenolone to the urinary 17-ketosteroids. The plasma level of dehydroisoandrosterone in the patient with adrenal carcinoma was approximately 1100 μg per 100 ml as compared to 100 μg per 100 ml in the normal subject.

The object of the studies to be described was to attempt to determine experimentally the effect of the level of circulating dehydroisoandrosterone on the transformations of labelled 17α-hydroxypregnenolone. The conversion of 17α-hydroxy pregnenolone to urinary 17-ketosteroids by a subject lacking pituitary, adrenals and gonads was also measured. The object of this study was to determine if the presence of these endocrine tissues was necessary for these conversions.

EXPERIMENTAL

METHODS

Determination of Radioactivity in Samples

Aliquots of samples to be counted were evaporated under nitrogen in 5 dram vials (Wheaton Glass Company) and dissolved in 5 ml toluene containing 0.3 per cent of 2,5-diphenyloxazole and 0.01 per cent of 1,4-bis-2(phenyloxazolyl)-benzene. Compounds insoluble in toluene were dissolved in 0.1 or 0.2 ml methanol prior to the addition of the toluene counting mixture. To correct for quenching encountered in the neutral extracts and in these samples first dissolved in methanol, an internal standard in 0.3 ml of toluene was added and the vials were recounted.

A Packard Tri-Carb Liquid Scintillation Spectrophotometer (Model 314-X) was used for counting. Tritium was counted with the photomultiplier set at voltage tap 6 (1,050 volts) and the pulse height discriminators set at 10 and 100. The efficiency for tritium at these settings was approximately 16 per cent. Carbon-14 was counted with the photomultiplier set at voltage tap 3 (745 volts) and the pulse height discriminators set at 10 and 100. The efficiency for carbon-14 counting at these settings was approximately 60 per cent.

For the simultaneous counting of tritium and carbon-14 the photomultiplier voltage was set at tap 5 (1,000 volts) with the pulse height discriminators set at 10 and 95. Under these conditions most of the tritium counts appeared in the first channel (10 to 95). Approximately two thirds of the C^{14} counts appeared in the second channel (95 to ∞) and the other third appeared in the first channel.

The total tritium and carbon-14 counts were calculated using the discriminator ratio method of Okita et al (194) as modified by Ulick (195). The following equations were used:

$$H^3 = N_1 - \frac{N_2}{b}$$

and $$C^{14} = N_2 - a\,N_1$$

where N_1 = total counts in first channel

N_2 = total counts in second channel

$$a = \frac{H^3 \text{ in second channel}}{H^3 \text{ in first channel}}$$

$$b = \frac{C^{14} \text{ in second channel}}{C^{14} \text{ in first channel.}}$$

On our liquid scintillation spectrometer, the optimum conditions for double label counting were obtained when the 'a' ratio was maintained between 0.018 and 0.024 and the 'b' ratio was between 1.7 and 2.2. Minor variations in high voltage tap settings and pulse height discriminator settings were made from time to time to maintain these optimal counting conditions.

For most of the samples counted, a 20-minute counting time was sufficient to give a standard error of 2 per cent. Where the ratio of total counts to background counts was low, a longer counting time was employed in order to maintain a standard error of at least 5 per cent.

The specific activity of a compound was considered to be established when the difference between the values for the final crystals and mother liquor was 10 per cent or less.

Melting Points and Infrared Analysis

All melting points were taken on a Kofler Block (H.O. Post

Scientific Instrument Co. Inc.). Infrared spectra were done on a Perkin—Elmer Model 221 Spectrophotometer. For infrared analysis the steroids were prepared either in CS_2 solution or as potassium bromide discs.

Paper Chromatography

Strips of Whatman No. 1 filter paper 15 x 42 cm were used. The solvent systems used in this study were (1) ligroin B:methanol:water (10:9:1) and (2) ligroin C : propylene glycol. In the ligroin B: methanol:water system the papers were first equilibrated in an atmosphere of the mobile and stationary phases for 4 hours. The chromatograms were then developed with mobile phase. The ligroin C : propylene glycol system was run as described by Savard (196). The papers were impregnated with propylene glycol : methanol (1:1) and blotted just prior to application of the samples. The papers were not equilibrated with the solvents prior to running in this system.

The 17-ketosteroids run as standards on the chromatograms were located by the method of Savard (196), whereby the standard strip was dipped in 2.5 N KOH in absolute ethanol, blotted, dipped in 2 per cent m.-dinitrobenzene in absolute ethanol, blotted and heated in an oven to 60-70°C. The 17-ketosteroids appeared as violet spots. This method is a modification of the Zimmerman reaction.

Compounds were eluted from the paper chromatograms by cutting the desired area into small squares and immersing them in methanol overnight. The methanol was then separated from the paper by filtering through a sintered glass funnel. The paper squares were rinsed several

times with methanol. The first methanol and the rinses were combined and evaporated to dryness on the flash evaporator.

Determination of Plasma Levels of Dehydroisoandrosterone and Androsterone

The plasma concentrations of dehydroisoandrosterone and androsterone were determined by the method of Burstein and Lieberman (190). The blood samples were collected in tubes containing potassium oxalate crystals. They were centrifuged immediately and the plasmas were transferred to measuring cylinders.

The plasma from each sample was deproteinized by shaking with three times its volume of freshly distilled tetrahydrofuran. Following the addition of a weight of ammonium sulphate equivalent to 80 per cent of the plasma volume, the mixture was shaken again. It was then filtered through Whatman No. 1 filter paper in a Buchner funnel. The filtrate was transferred to a separatory funnel and the lower phase was discarded. The upper (tetrahydrofuran) phase was filtered through glass wool and 0.09 ml of 70 per cent percholoric acid per 100 ml tetrahydrofuran added. The tetrahydrofuran was then placed overnight in an incubator at 37°C. The tetrahydrofuran extract was neutralized with concentrated ammonium hydroxide and then evaporated on the flash evaporator. The residual aqueous phase was transferred to a separatory funnel with 100 ml. of ethyl acetate and 10 ml of 0.1 N NaOH and shaken. The lower phase was discarded. The ethyl acetate was washed three times by shaking with distilled water, dried over sodium sulphate and evaporated to dryness on the flash evaporator.

The dry extract was chromatographed on a 2 gm silica gel column.

The column was prepared in distilled methylene chloride and then washed with 30 to 40 ml of the same solvent. The extract was transferred to the column in methylene chloride. The column was then developed with the following fractions: (1) 60 ml of methylene chloride; (2) 40 ml of 0.5 per cent ethanol in methylene chloride; (3) 20 ml of a 1 per cent ethanol in methylene chloride; (4) 20 ml of 2 per cent ethanol in methylene chloride; (5) 20 ml of 3 per cent ethanol in methylene chloride and (6) 20 ml of 10 per cent ethanol in methylene chloride. The fractions were taken to dryness under vacuum. Fractions 3, 4 and 5 were combined and chromatographed on paper in the ligroin B : methanol water system for 10 hours with dehydroisoandrosterone and androsterone as standards. The areas on the paper corresponding in mobility to the two standards were eluted separately by cutting each into small squares and allowing them to stand overnight in methanol. Aliquots of each eluate were then removed for micro—Zimmerman determination.

For the colorimetric determination of the dehydroisoandrosterone and androsterone, the aliquots of the paper chromatogram eluates were evaporated under a stream of nitrogen and to each was added 0.1 ml absolute ethanol, 0.1 ml 2 per cent dinitrobenzene in absolute ethanol and 0.1 ml 5 N KOH in water. After mixing, the tubes were placed in the dark for 1 hour. Distilled water (0.3) and freshly distilled tetrahydrofuran (1.5 ml) were added to each tube. The tubes were then shaken and the upper phase was filtered through 2 ml syringes into 0.8 ml microcuvettes. The needles of the syringes had cotton placed in

them. The optical densities of each sample were then read in a Beckman DU Spectrophotometer at 440, 520 and 600 mμ. The corrected optical density was thencalculated using the Allen equation (198):

Corrected optical density

= optical density at 520 mμ

- ½ (optical density at 600 mμ + optical density at 420 mμ).

Simultaneously with the colorimetric determination of the plasma samples dehydroisoandrosterone standards of 15, 30 and 45 μg were also processed as described for the unknown.

Hydrolysis of Urinary Conjugates and Extraction of Steroids

In order to hydrolyse the steroid glucosiduronidates, each 24 hour urine collection was first adjusted to pH 5.0 with 50 per cent sulphuric acid. Five per cent by volume of 2 M acetate buffer pH 5.0, 10 ml chloroform and 500 units/ml of β-glucuronidase (Ketodase, obtained from Warner-Lambert, Canada Limited) were added to each urine specimen. Following incubation at 37°C for 5 days, each urine was extracted with ethyl acetate (1 x 1 volume + 2 x ½ volume). The combined ethyl acetate extracts were washed with 25 ml aliquots of 1 N NaOH until no further pigments were being removed and then with distilled water until neutral. The ethyl acetate was dried over anhydrous sodium sulphate and evaporated to dryness on a flash evaporator. This extract contained the steroids excreted in the free form plus those excreted as glucosiduronidates.

The combined aqueous phases (urine plus NaOH and water washes)

were then processed by the solvolysis procedure of Burstein and Lieberman (199) which cleaves the sulphuric acid conjugates of the steroids. The urine plus washes were adjusted to pH 1 with sulphuric acid. Twenty per cent by volume of sodium chloride was added to each specimen and the urine was then extracted in five parts with an equal volume of tetrahydrofuran (refluxed and freshly distilled over potassium hydroxide pellets). The tetrahydrofuran solution was filtered through glass wool and 0.09 ml of 70 per cent perchloric acid per 100 ml of tetrahydrofuran were added. After incubation overnight at 37°C., 5 ml of concentrated ammonium hydroxide per liter of tetrahydrofuran were added and the organic solvent was then removed on the flash evaporator. The residual aqueous fraction was transferred to a separatory funnel with 50 ml of 0.1 N NaOH. It was extracted with ethyl acetate (300 + 2 x 200 ml). The combined ethyl acetate extracts were washed with 1 N NaOH solution and distilled water until neutral, dried over anhydrous sodium sulphate and evaporated to dryness on the flash evaporator. This extract contained the steroids excreted as sulphates.

Chromatography of Extracts

The extracts were first chromatographed on silica gel columns prepared in methylene chloride. This initial column separated the 17-ketosteroids from the more polar metabolites of 17α-hydroxypregnenolone (e.g. pregnenetriols). The column was developed first with methylene chloride followed by solutions of 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 20 and 50 per cent ethanol in methylene chloride in that order. An aliquot of each fraction was removed for radioactivity determination.

The 17-ketosteroids were eluted by 3 and 4 per cent ethanol. The fractions from each column were combined according to the radioactivity content.

The 17-ketosteroid fractions from the silica gel column were then chromatographed on alumina using the gradient elution technique of Lakshmanan and Lieberman (200). Neutral Woelm alumina containing 5 per cent (v/w) of water was used. A 15 gram column was prepared in benzene. The solvent reservoir for the column contained 700 ml of benzene. A dropping funnel attached to the top of the reservoir contained 4 per cent ethanol in benzene. The apparatus used was that designed by the originators of the method. Air pressure was used to give a flow rate of 2.5 ml per minute. Ten ml fractions were collected from tubes 1 to 20 and tubes 61 to 100. Five ml fractions were collected from tubes 21 to 60. The Zimmerman reaction was performed by the method of Holtorff and Koch (201) on one-tenth of each fraction after it was taken to dryness. The column fractions were then combined into groups according to the pattern of elution of Zimmerman chromogens and evaporated to dryness. Aliquots were removed for counting. The dehydroisoandrosterone, androsterone and etiocholanolone as obtained from the gradient elution column (the sequence of elution of the different 17-ketosteroids was known from runs with standards) were further purified by chromatography on paper in the Skelly Solve C/propylene-glycol system. The 17-ketosteroids (a maximum of 12 mg per paper) were applied to a 13 cm starting line. The chromatograms were developed for 24 hours with Skelly Solve C and dried overnight at room temperature in a fume hood. A 3.4 mm strip was then cut from the center of the chromatogram and stained by the Zimmerman

reaction as applied to paper. The area on the remainder of the chromatogram corresponding to the Zimmerman positive material was eluted. To remove the last traces of propylene glycol from the samples, they were partitioned between ethyl acetate and distilled water. The samples were dissolved in 200 ml of ethyl acetate and the solution was washed three times with distilled water (20 ml + 2 x 10 ml). The ethyl acetate was dried over sodium sulphate and evaporated to dryness on the flash evaporator.

To facilitate crystallization of the individual 17-ketosteroids at this stage they were percolated through small alumina columns prepared in benzene : ligroin B (1:1) Harshaw alumina (200 mesh) which had been deactivated by the procedure as described by Solomon et al (119) was used. The alumina was refluxed with ethyl acetate for 5 days, the solvent was removed by filtration. The alumina was washed further with methanol, then continuously with water for 2 days and again with methanol. The alumina was dried overnight at 100 — 120°C. and deactivated by the addition of 5 ml of water per 100 gm. It was stored in tightly stoppered bottles. The weight of alumina used was 100 times that of the weight of steroid being purified. The smallest columns used contained 1 gm of alumina. The columns were developed with ligroin B : benzene (1:1), ligroin B : benzene (1:4), benzene, benzene : ether (99:1), benzene-ether (98:2), benzene : ether (96:4) and benzene : ether (9:1). The 17-ketosteroids were eluted in the first three benzene:ether mixtures. The column fractions were evaporated under vacuum and combined after a visual examination of the residue in each

flask. The 17-ketosteroids were usually crystalline at this stage.

Preparation of 17-Ketosteroid Derivatives

The acetates were prepared by dissolving the steroids in pyridine and acetic anhydride. The pyridine and acetic anhydride were used in a ratio of 2:1. A minimum of 0.2 molar excess of acetic anhydride per mole of steroid was used. The minimum volumes of reagents used were 0.2 ml pyridine plus 0.1 ml acetic anhydride. After standing overnight at room temperature, either the excess acetic anhydride and pyridine were removed under a stream of nitrogen or the reaction mixture was added to 100 ml of ice water containing 5 ml 6 N H_2SO_4, which was extracted four times with 50 ml ethyl acetate. The combined ethyl acetate extracts were washed with 6 N H_2SO_4 (5 x 5 ml), 1 N NaOH (3x 5 ml) and distilled water (three times), dried over anhydrous sodium sulphate and evaporated to dryness on the flash evaporator. The acetates were then percolated through small alumina columns which were prepared in ligroin B : benzene (4:1). The columns were then developed with ligroin B : benzene mixtures (7:3, 6:4, 1:1, 4:6, 3:7, 1:4, 1:9), in that order, followed by benzene. The acetates were eluted by the last three ligroin B :benzene mixtures and the benzene. The fractions containing the steroid acetate (as determined visually) were combined.

Benzoates were formed by treating the steroid dissolved in pyridine, with a minimum of a 0.2 molar excess of benzoyl chloride. The reaction was performed overnight at room temperature. The reaction mixture was then poured into ice water and processed in the manner described for the acetylations. The resulting extracts were chromatographed on alumina and then crystallized several times.

One of the derivatives of dehydroisoandrosterone was the 3-acetate-5,6α-epoxide formed from the 3-acetate. The dehydroisoandrosterone acetate was dissolved in 1 ml of chloroform and treated with 20 ml of a solution of perbenzoic acid (8.2 mg/ml) in benzene. The mixture was placed in the refrigerator for 70 hours and then added to 100 ml of distilled water. The steroid was extracted with ethyl acetate (6 x 40 ml). The combined ethyl acetate extracts were washed with 1 N NaOH (3 x 10 ml) and distilled water (three times), dried over anhydrous sodium sulphate and evaporated to dryness on the flash evaporator. The dry extract was chromatographed on an alumina column prepared in ligroin B : benzene (4:1). The column was developed with ligroin B : benzene mixtures (4:1, 3:2, 1:4), benzene and benzene : ether mixtures (199:1, 99:1, 98:2, 95:5, 9:1, 4:1, 1:1) and ether. The dehydroisoandrosterone-3-acetate-5,6α-epoxide was eluted in a large number of fractions by the benzene : ether mixtures. The fractions containing crystalline material were combined and crystallized several times.

The perbenzoic acid used for the formation of epoxides was prepared from benzoyl peroxide (202). A total of 5.2 gm of sodium was dissolved in 100 ml of absolute methanol with cooling. The resulting sodium methoxide was cooled by immersion in a dry ice - acetone freezing mixture. A solution of 50 gm of benzoyl peroxide in 200 ml of dry chloroform was prepared and cooled to 0°. The sodium methoxide was added immediately with shaking and cooling so that the temperature did not rise above 0°. The mixture was kept for 4 -

5 minutes in an ice-salt bath with continuous shaking and transferred to a separatory funnel where it was rapidly extracted with 500 ml of water (containing ice) to obtain sodium perbenzoate. The aqueous layer was extracted twice with 100 ml portions of cold chloroform to remove methyl benzoate. The perbenzoic acid was formed from the sodium perbenzoate in the aqueous solution by the addition of 225 ml cold 1 N H_2SO_4. The acid was extracted from solution with cold benzene (3 x 100 ml). The benzene solution was used for the oxidations. The solution of perbenzoic acid was standardized against 0.1 N sodium thiosulphate. The 0.1 N sodium thiosulphate was prepared by dissolving 49.6 gm $Na_2S_2O_3.5H_2O$ and 1.6 gm NaOH in water and the solution was diluted to a volume of 4 liters. The sodium thiosulphate solution was standardized against potassium biniodate. Twenty-five ml of 0.1 N $KIO_3.HIO_3$, 2 gm KI, 5 ml concentrated HCl and 200 ml of water were titrated with the sodium thiosulphate solution using chloroform as an indicator. The amount of active oxygen in the perbenzoic acid was estimated by iodometric titration. One gram of sodium iodide was dissolved in 50 ml of water. Five ml of glacial acetic acid and 5 ml of chloroform were added. One ml of the perbenzoic acid solution was added. The liberated iodine was titrated with the sodium thiosulphate solution.

Sodium borohydride reductions were performed as described by Norymberski and Woods (203). A 0.4 per cent solution of the steroid in methanol was prepared. After the addition of 1.6 moles of solid sodium borohydride per mole of steroid, the mixture was kept at 0°C

for 1 hour. Several drops of acetic acid were added to stop the reaction and the methanol was removed under vacuum on the flash evaporator. The residue was dissolved in 100 ml of ethyl acetate which was then washed with 0.1 N NaOH (3 x 5 ml) and distilled water (3 times), dried over anhydrous sodium sulphate and evaporated to dryness on the flash evaporator. Following chromatography on an alumina column the steroid derivative was crystallized several times.

Check of Purity of 17α-Hydroxypregnenolone-7α-H^3

The 17α-hydroxypregnenolone-7α-H^3 (2.56 μc/μg) used for these experiments was purchased from the New England Nuclear Corporation. On arrival, it was stored in benzene—ethanol (3:2). Aliquots of this solution were further diluted with methanol for counting.

To test the homogeneity of the 17α-hydroxypregnenolone-7α-H^3, a total of 1.57×10^6 cpm was mixed with 99.6 mg of non—radioactive 17α-hydroxypregnenolone and the mixture was acetylated overnight with pyridine and acetic anhydride. The resulting 3-monoacetate was crystallized three times using acetone and acetone:methanol mixtures. Following each crystallization, approximately 1 mg of the crystals were removed and accurately weighed on a Mettler microbalance before transfer to a vial for counting. The unfractionated mother liquors were accurately weighed, dissolved in a known volume of methanol and an aliquot of the solution corresponding to approximately 1 mg of steroid was removed for counting. The specific activities (cpm/mg) of the crystals and mother liquors from each crystallization were determined and compared to the expected value.

Administration of 17α-Hydroxypregnenolone—7α-H^3 to Subjects

In the preparation of the 17α-hydroxypregnenolone-7α-H^3 for administration to the subjects under study, a known number of counts (approximately 8 x 10^6 cpm) was taken to dryness in a sterile vial under a stream of nitrogen. For injection the material was dissolved in 1 ml of absolute ethanol and diluted with 15 ml of sterile saline. It was then administered intravenously over a period of 3 — 5 minutes. Blood was twice drawn back into the syringe and re-injected. Following the injection, the vial, syringe and needle were washed with saline and ethyl acetate. The combined ethyl acetate extracts were washed three times with water and dried over sodium sulphate and evaporated to dryness on a flash evaporator. Aliquots of this extract were removed for radioactivity determination. The number of counts in the syringe wash was subtracted from the number originally prepared for the injection to determine the number of counts actually administered.

EXPERIMENTAL DESIGN AND SUBJECTS

Two subjects were used for these experiments. For the experiments planned to study the effect of an increased plasma level of dehydroisoandrosterone on the conversion of 17α-hydroxypregnenolone to urinary 11-desoxy-17-ketosteroids, the subject was a 37-year-old normal male who was excreting 12.1 mg of 17-ketosteroids per day. This value was obtained by summing the individual excretions of the 17-ketosteroids which were separated by a gradient elution column and assayed by the Zimmerman reaction.

In the first control experiment (Experiment I) the subject received only an intravenous injection of 17∝-hydroxypregnenolone-7∝-H^3. In the second experiment (Experiment II) the same subject was given 100 mg of dehydroisoandrosterone per os at hourly intervals for a period of 14 hours. Blood samples were drawn at 0, 4, 8 and 13 hours. The plasma concentrations of dehydroisoandrosterone and androsterone in these samples were determined. The same amount of labelled 17∝-hydroxypregnenolone was given at the eighth hour. Experiment III was performed to study the effect of the absence of certain endocrine tissues on the conversion of 17∝-hydroxypregnenolone to urinary 17-ketosteroids. The subject was a patient with breast cancer who had been hypophysectomized, adrenalectomized and ovariectomized. She was injected with 17∝-hydroxypregnenolone-7∝-H^3.

In all three experments, complete 24-hour urine collections were made for the four days following the administration of the labelled 17∝-hydroxypregnenolone. Dehydroisoandrosterone, androsterone and etiocholanolone were then isolated from these urines and the quantity of each as well as the amount of radioactivity in each of the three metabolites were determined.

The urines were first treated with β-glucuronidase to hydrolyse the steroid glucosiduronidates and then subjected to the solvolysis procedure to hydrolyse the steroid sulphates. In Experiment III an ethyl acetate extraction was not done on the urines following the β-glucuronidase hydrolysis. The urine was solvolysed directly. In this instance, the steroids liberated by the β-glucuronidase and the steroids released by solvolysis were recovered together in the final

extract. Aliquots of each extract in each experiment were removed for counting.

The urine extracts for the three experiments were combined as follows:-

Experiments I and II: For each of the two experiments the extracts obtained after β-glucuronidase treatment and those obtained after solvolysis were processed separately. Experiment III: The extracts which contained the steroids excreted as the glucosiduronidates and as the sulphates during the 4 days of urine collection were combined and 9.64 mg of dehydroisoandrosterone, 9.438 mg of androsterone and 9.806 mg of etiocholanolone were added to the combined extracts to facilitate isolation of the excreted metabolites.

Following the chromatography of the extracts on the silica gel columns, the 17-ketosteroid eluates resulting from β-glucuronidase hydrolysis and solvolysis were combined in Experiment I, while in Experiment II they were not combined as it was anticipated that large amounts of urinary dehydroisoandrosterone would be present in the solvolysis extract. However, the corresponding ketosteroids from the two extracts in Experiment II were combined after paper chromatography. After the paper chromatography step in Experiments I and II, aliquots were removed from the three 17-ketosteroid fractions for radioactivity and micro-Zimmerman quantitation.

Following the percolation of the steroids through the small alumina columns, they were crystallized several times and the specific activities of the crystals and unfractionated mother liquors were determined as described above in the determination of the purity of the

17α-hydroxypregnenolone-7α-H^3. When it was apparent that homogeneity had been established by this process, i.e. the specific activities of the crystals and mother liquor from the final crystallization agreed within the limits of experimental error, the 3—acetate of each of the 17-ketosteroids was formed. In Experiments I and III carrier steroid was added to a known number of counts prior to the formation of the acetate. In Experiment I, 30.4 mg carrier dehydroisoandrosterone was added to 3,220 cpm, 69.7 mg androsterone was added to 16,200 cpm and 49.8 mg etiocholanolone was added to 9,360 cpm. In Experiment III, 8.116 mg androsterone were added to 1,433 cpm and 12.021 mg etiocholanolone were added to 3,889m cpm.

The acetates of the three 17-ketosteroids in Experiments I and II were prepared and crystallized several times until the specific activities of the crystals and unfractionated mother liquors were the same within the limits of experimental error. In Experiment III further derivatives were prepared from the 17-ketosteroid acetates prior to crystallizations.

In Experiments I and II, a second derivative of each of the three 17-ketosteroids was prepared. In the case of dehydroisoandrosterone, the 3-acetate-5,6,α-epoxide was formed from the 3-acetate. The androsterone acetate samples in the three experiments were converted to the 3α,17β-diol-3acetate derivative with sodium borohydrode.

The etiocholanolone-3—acetates from the three experiments were reduced with sodium borohydride, following which the 17—benzoate was prepared.

The minimal percent conversions of the injected 17α-hydroxypregnenolone to each of the 11-desoxy-17-ketosteroids was calculated using the relationship:-

$$\% \text{ Conversion} = \frac{KS_k \times 100}{\text{cpm of injected steroid}}$$

where K = excretion of steroid over the 4-day peroid of the study

S_k = specific activity of isolated K.

RESULTS

PURITY OF 17α-HYDROXYPREGNENOLONE-7α-H^3

That the 17α-hydroxypregnenolone--7α-H^3 used for these experiments was at least 97 per cent pure can be surmised from Table II. A total of 1.57×10^6 cpm of 17α-hydroxypregnenolone-7α-H^3 plus 99.6 mg of non-radioactive carrier was acetylated and the resulting 17α-hydroxypregnenolone acetate was crystallized three times. The specific activities of the crystals and unfractionated mother liquors are presented in the table. These specific activities did not differ significantly in the crystals and mother liquors, and were the same as the calculated specific activity within the limits of experimental error.

PLASMA CONCENTRATIONS OF DEHYDROISOANDROSTERONE AND ANDROSTERONE AFTER DEHYDROISOANDROSTERONE FEEDING IN EXPERIMENT II.

Prior to Experiment II, it had been determined experimentally that the plasma level of dehydroisoandrosterone could be elevated by

TABLE II

Test for homogeneity of 17α-hydroxypregnenolone-7α-H^3

Crystallization	Solvent used	Specific activity cpm/mg	
		Crystals	Mother Liquor
1	acetone	13,620	12,580
2	acetone-methanol	13,930	14,320
3	acetone-methanol	13,400	13,360
Calculated		13,920	

the hourly administration per os of 100 mg quantities of dehydroisoandrosterone. The peak level of dehydroisoandrosterone was obtained at the eighth hour. The plasma levels of dehydroisoandrosterone, and androsterone at various intervals during Experiment II are shown in Table III. In contrast to the trial experiment, the peak level of dehydroisoandrosterone in Experiment II occurred at the fourth hour. The peak plasma level of androsterone was at the eighth hour. The labelled 17α-hydroxypregnenolone was administered at the eighth hour.

URINARY EXCRETION OF RADIOACTIVITY.

The percentages of the administered radioactivity extracted from the urine after hydrolysis of the conjugates with β-glucuronidase and by solvolysis in experiments I and II are shown in Table IV. The total radioactivity extracted from the urine following the hydrolytic procedures was 38.6 and 79.9 per cent for Experiments I and II respectively. Almost the entire difference between the two results was in the Day 1 extract obtained following solvolysis. In Experiment I, more radioactivity was extracted following β-glucuronidase treatment than after solvolysis. The reverse was found for Experiment II. The urines examined in Experiment III were first treated with β-glucuronidase and then extracted with tetrahydrofuran. The solvolysis procedure was performed on this solution. Following this treatment the final ethyl acetate extract contained the free steroids which were originally glucosiduronidates and sulphates. The per cent of the administered radioactivity extracted from each day's urine in Experiment

TABLE III

Plasma Concentration of 17-Ketosteroids after the Administration of 12 x 100 mg of Dehydroisoandrosterone per os During 14 hours (Experiment II)

Time (hr)	Dehydroisoandrosterone (µg/100 ml plasma)	Androsterone (ug/100 ml plasma)	Total
0	122	36	158
4	511	54	565
8*	324	96	420
13	273	32	305

* 17α-hydroxypregnenolone-7α-H^3 administered.

III is shown in Table V. A total of 28.5 per cent of the administered radioactivity was extracted from the four-day urine collection for Experiment III. This result is approximately 10 per cent lower than the corresponding figure for Experiment 1 . In none of the three experiments was more than 1 per cent of the administered radioactivity extracted from the fourth day urine collection. This indicates that the period of urine collection for all three experiments was adequate.

PATTERNS OF ELUTION OF RADIOACTIVITY FROM SILICA GEL COLUMNS

In Experiments I and II the extracts obtained following β-glucuronidase hydrolysis and following solvolysis were chromatographed separately on silica gel columns. The patterns of elution of the radioactivity from these 4 columns and the one for Experiment III were similar. The sole exception was the pattern obtained for the glucosiduronidate extract of Experiment I where it would appear that the first two major peaks did not separate as they did in the chromatography of the other extracts. These radioactivity elution patterns for the silica gel columns are shown in Figures IV, V and VA. The radioactivity in each of the combined column fractions are shown in Tables VI, VII and VIII. In Experiment I, 89.1 per cent of the radioactivity extracted from the urine was recovered from the two silica gel columns. The corresponding recovery figures for Experiments II and III were 66.5 and 98.2 per cent, respectively. The first peak of radioactivity eluted from the column contained the 11-desoxy-17-ketosteroids in each instance (e.g. the 17-ketosteroids were isolated from fractions 13 to 23 of the silica gel column used for the extract obtained after

TABLE IV

RADIOACTIVITY EXTRACTED FROM URINE AFTER β-GLUCURONIDASE HYDROLYSIS AND SOLVOLYSIS IN EXPERIMENTS I AND II

Day	β-Glucuronidase Hydrolysis		Solvolysis		Enzyme and Solvolysis	
	Radioactivity 10^3 cpm	% of Injected Dose	Radioactivity 10^3 cpm	% of Injected Dose	Radioactivity 10^3 cpm	%of Injected Dose
			Experiment I (8.13×10^6 cpm injected)			
1	1,588	19.5	574	7.1	2,162	26.6
2	426	5.2	208	2.6	634	7.8
3	163	2.0	102	1.3	265	3.3
4	39	0.5	29	0.4	68	0.9
Total	2,216	27.2	913	11.4	3,129	38.6
			Experiment II (8.29×10^6 cpm injected)			
1	2,074	25.0	3,700	44.6	5,774	69.6
2	415	5.0	181	2.2	596	7.2
3	126	1.5	54	0.7	180	2.2
4	52	0.6	25	0.3	77	0.9
Total	2,667	32.1	3,960	47.8	6,627	79.9

TABLE V

Radioactivity Extracted From Urine After β-Glucuronidase Hydrolysis and Solvolysis in Experiment III (8.11×10^6 cpm Injected)

Day	Enzyme and Solvolysis	
	Radioactivity 10^3 cpm	% of Injected Dose
1	1,313	16.2
2	749	9.2
3	179	2.2
4	73	0.9
Total	2,314	28.5

β-glucuronidase treatment in Experiment I).

SPECIFIC ACTIVITIES OF THE 17-KETOSTEROIDS AND THEIR DERIVATIVES

After the initial silica gel column, the 11-desoxy-17-ketosteroids were separated and purified by gradient elution chromatography and paper chromatography. The individual steroids were then percolated through small alumina columns and crystalized to constant specific activity. At this stage, carrier steroid was added to a known number of counts of each of the three 17-ketosteroids in Experiment I and the androsterone in Experiment II. This was done in order that the derivatives formed could be crystallized several times and that there would be enough material to permit the removal of adequate aliquots for counting. In Experiment I, 30.4 mg carrier dehydroisoandrosterone was added to 3,220 cpm, 69.7 mg androsterone was added to 16,200 cpm and 49.8 mg etiocholanolone was added to 9,360 cpm. In Experiment II, 29.9 mg carrier androsterone were added to 31,192 cpm. The acetates of the 17-ketosteroids were then prepared and in Experiments I and II these were crystallized several times. A second derivative was then prepared from each of the acetates in the first two experiments. These were dehydroisoandrosterone-3-acetate-5,6α-epoxide, androstane-3α,17β-diol-3-acetate and etiocholane--3α,17β-diol-3-acetate-17-benzoate. The second derivative was crystallized several times in each instance. The specific activities of the crystals and unfractionated mother liquors for the free compounds and their derivatives for Experiments I and II are shown in Tables IX and X, respectively.

As can be seen in Table IX the specific activities of the

Figure IV.

ELUTION PATTERNS OF RADIOACTIVITY FROM SILICA GEL COLUMNS OF EXPERIMENT I.

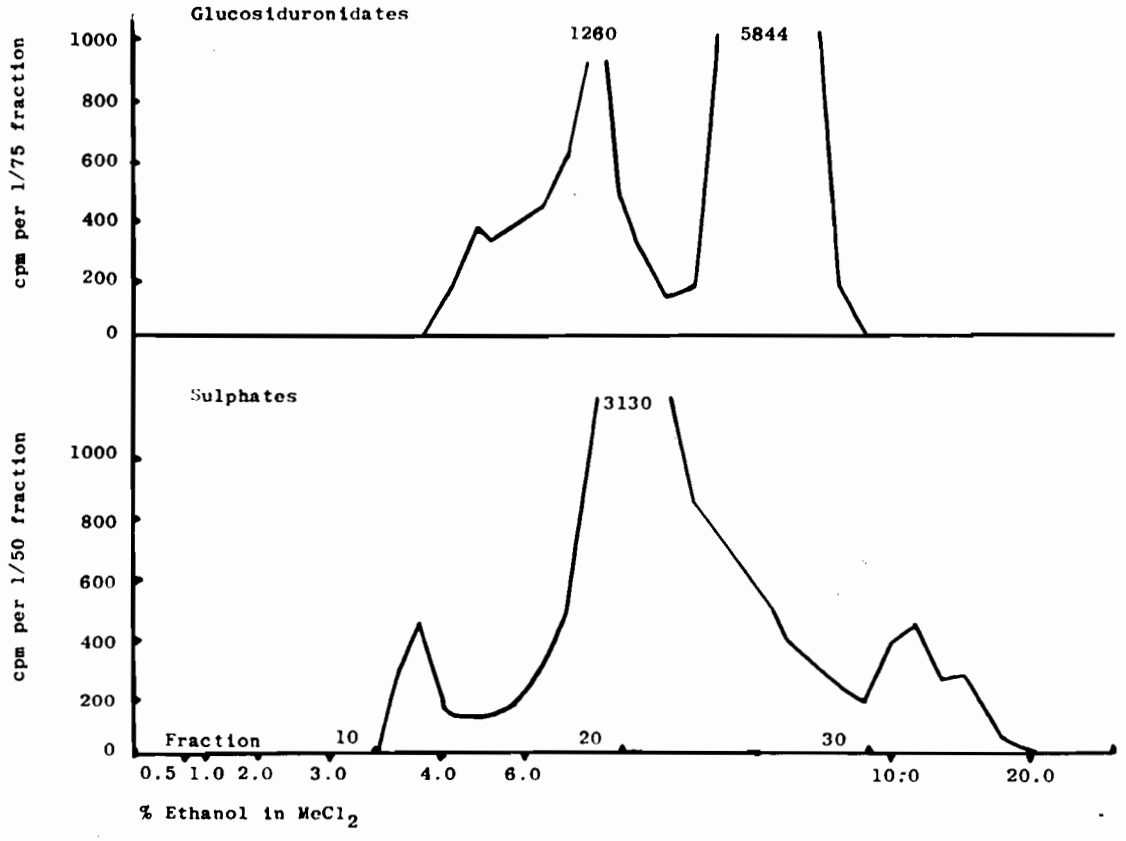


Figure V.

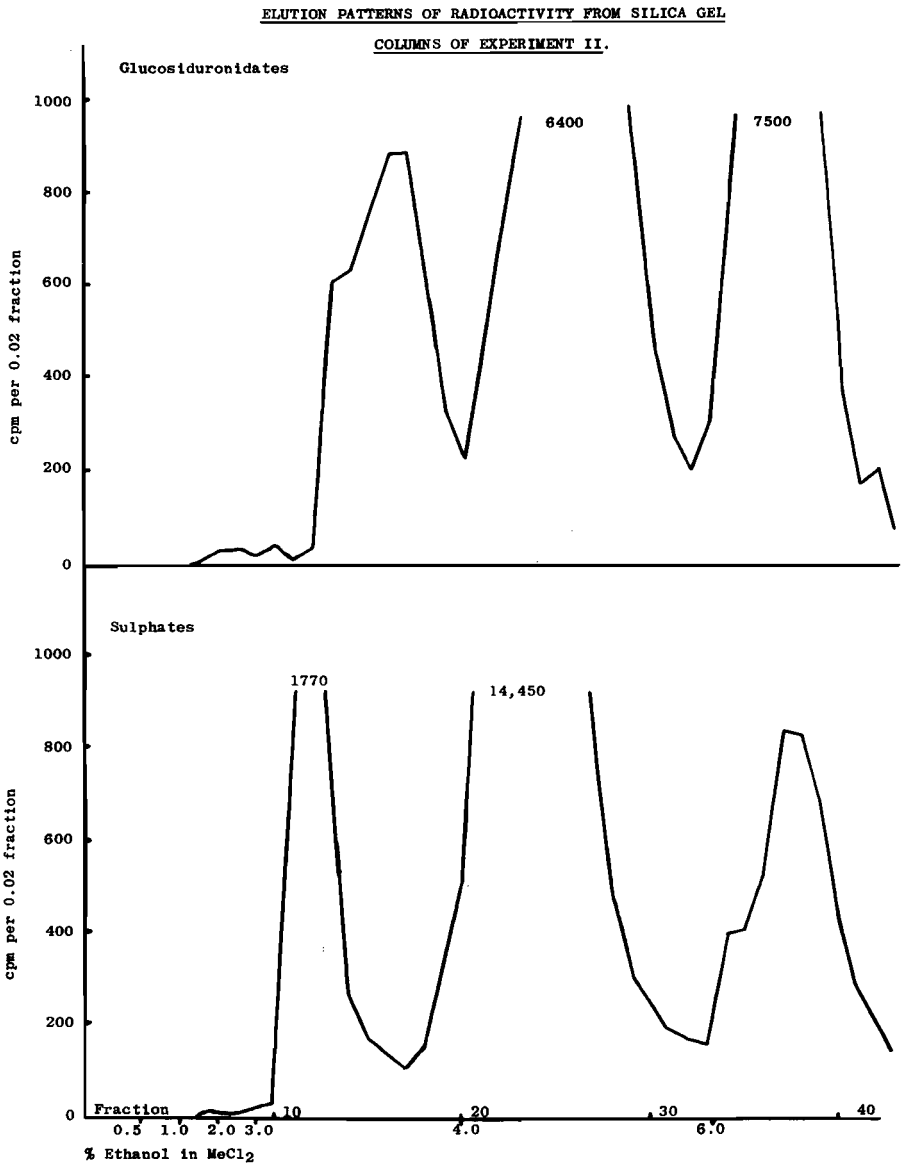

ELUTION PATTERNS OF RADIOACTIVITY FROM SILICA GEL
COLUMNS OF EXPERIMENT II.
Glucosiduronidates
6400
7500
cpm per 0.02 fraction
1000
800
600
400
200
0
Sulphates
1770
14,450
cpm per 0.02 fraction
Fraction
10
20
30
40
0.5
1.0
2.0
3.0
4.0
6.0
% Ethanol in MeCl2

Figure VA

ELUTION PATTERN OF RADIOACTIVITY FROM SILICA GEL COLUMNS OF EXPERIMENT III.

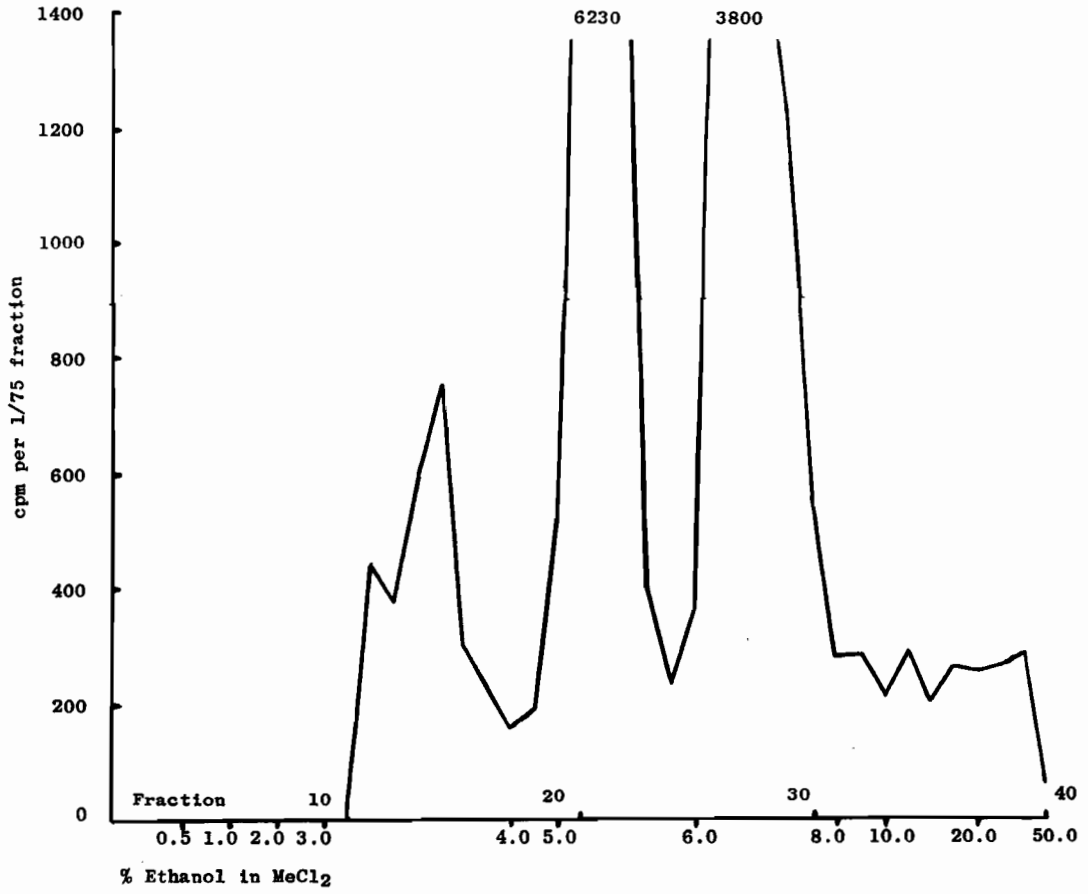


TABLE VI

RADIOACTIVITY IN FRACTIONS COMBINED FROM SILICA GEL COLUMNS FOR EXPERIMENT I

Fractions	Radioactivity	
	cpm (10^3)	% of injected dose
	β-Glucuronidase Extract	
1 - 12	0	0
13 - 23	322.0	3.96
24 - 29	1529.0	18.81
Total	1851.0	22.77
	Solvolysis Extract	
1 - 10	0	0
11 - 15	66.4	0.82
16 - 30	781.5	9.61
31 - 38	73.6	0.91
Total	921.5	11.34

TABLE VII

RADIOACTIVITY IN FRACTIONS COMBINED FOR SILICA GEL COLUMNS FOR EXPERIMENT II

Fractions	Radioactivity	
	cpm (10^3)	% of injected dose
	β-Glucuronidase Extract	
1 - 12	2.1	0.03
13 - 20	191.8	2.31
21 - 32	957.0	11.54
33 - 46	965.4	11.65
Total	2116.3	25.52
	Solvolysis Extract	
1 - 9	1.3	0.02
10 - 17	150.0	1.81
18 - 32	1931.8	23.30
32 - 45	209.1	2.52
Total	2292.2	27.65

TABLE VIII

RADIOACTIVITY IN FRACTIONS COMBINED FROM SILICA GEL COLUMN FOR EXPERIMENT III

Fractions	Radioactivity	
	cpm (10^3)	% of injected dose
1 - 8	0	0
9 - 18	257.6	3.18
19 - 24	1014.3	12.51
25 - 32	890.4	10.98
33 - 41	108.9	1.34
Total	2271.2	28.01

TABLE IX

SPECIFIC ACTIVITIES OF THE ISOLATED 11-DESOXY-17-KS AND THEIR DERIVATIVES IN EXPERIMENT I

Steroid	Crystallization	Free Compound		First Derivative		Second Derivative	
		Crystals	ML*	Crystals	ML	Crystals	ML
				(cpm/mg)			
						3-acetate-5,6α-epoxide	
Dehydroisoandrosterone	1	1,560	726	66	222	48	44
	2	1,514	1,410	55	97	43	24
	3			55	76	43	40
	4			54	59		
	Calculated			86		52	
						3α,17β-diol-3-acetate	
Androsterone	1	2,345	2,115	184	271	171	163
	2	2,360	2,026	177	188	164	160
	3	2,430	2,530			168	168
	Calculated			185		177	
						3α-17β-diol-3-acetate-17-benzoate	
Etiocholanolone	1	2,170	1,102	146	137	114	92
	2	2,210	2,540	151	140	110	99
	3					107	102
	Calculated			149		115	

* Mother liquor

TABLE X

SPECIFIC ACTIVITIES OF THE ISOLATED 11-DESOXY-17-KETOSTEROIDS AND THEIR DERIVATIVES FOR EXPERIMENT II

Steroid	Crystal-lization	Free Compound		First Derivative		Second Derivative	
		Crystals	ML*	Crystals	ML	Crystals	ML
				(cpm/mg)			
				3-acetate		3-acetate-5,6α-epoxide	
Dehydroisoandrosterone	1	232	232	135	200	163	151
	2	232	245	181	212	160	158
	3			184	207	162	164
	Calculated			202		176	
						3α,17β-diol-3-acetate	
Androsterone	1	372	289	133	164	173	164
	2	378	364	164	155	175	176
	3			163	142	168	176
	4			167	167		
	Calculated			178		167	
						3α,17β-diol-3-acetate-17-benzoate	
Etiocholanolone	1	91	114	75	67	58	45
	2	90	99	77	63	57	56
	3			77	65	54	54
	Calculated			79		59	

* Mother liquor

crystals and mother liquors of the dehydroisoandrosterone agreed within the limits of experimental error after two crystalizations. However, on formation of the acetate four crystallizations were required to attain a constant specific activity. The specific activity of the crystals after the fourth crystallization was 37 per cent lower than the calculated value based on the addition of carrier. There was a further drop in the specific activity on formation of the α-epoxide but as only 3.9 mg of this derivative were obtained there was not enough material after three crystallizations for further analysis. Because the specific activity of the α-epoxide was lower than the calculated value, it was not certain whether radiochemical homogeneity had been established in this instance. In the cases of the androsterone and etiocholanolone the specific activities of both derivatives for each steroid did not differ significantly from the calculated specific activities. As is evident in Table X, no trouble was encountered in establishing the radiochemical homogeneity of the three metabolites in Experiment II.

In Experiments I and II specific activities of the 11-desoxy-17-ketosteroids were measured before and after paper chromatography using the micro-Zimmerman reaction on aliquots of the isolated steroids. These specific activities are shown in Table XI. The final specific activities of the free steroid as well as the final calculated specific activities are also shown in this Table. In Experiment II the specific activities of each of the three steroids in the extracts obtained after β-glucuronidase hydrolysis and after solvolysis were measured separately.

TABLE XI

SPECIFIC ACTIVITIES OF THE THREE 17—KETOSTEROIDS AT VARIOUS STAGES OF PURIFICATION IN EXPERIMENTS I AND II

	Dehydroisoandrosterone	Androsterone	Etiocholanolone
		(cpm/mg)	
		Experiment I	
Before paper chromatography	1416	2361	4100
After paper chromatography	1614	2290	2217
Final crystals of free compound	1514	2430	2210
Final calculated	798	2295	2098
		Experiment II	
Before paper chromatography			
β-glucuronidase	293	605	177
solvolysis	208	679	898
After paper chromatography	225	605	349
Final crystals of free compound	232	378	90
Final calculated	195	355	82

The final calculated specific activities were considerably lower than those obtained prior to paper chromatography in all cases except one.

In Experiment I, the specific activities of the androsterone obtained from the second derivative and that determined prior to paper chromatography did not differ significantly. The etiocholanolone in Experiment I was purified considerably by paper chromatography.

Following the crystallization of the free steroids reisolated in Experiment III, carrier steroid was added to a known number of counts. A total of 8.1 mg androsterone were added to 1,433 cpm and 12.0 mg etiocholanolone were added to 3,889 cpm. There was not enough steroid remaining after crystallization of the dehydroisoandrosterone for the preparation of a derivative. Because of the small quantities of steroid available in the cases of androsterone and etiocholanolone, the acetates were not crystallized. Instead, a second derivitive was prepared directly. These derivatives were androstane-3α,17β-diol-3-acetate from the androsterone acetate, and etiocholane-3α,17β-diol-3-acetate-17-benzoate from the etiocholanolone acetate. These derivatives were crystallized several times. The specific activities of the crystals and unfractionated mother liquors of the free compounds and the derivatives are shown in Table XII. The specific activity of the final crystals of the androsterone derivative was lower than the calculated specific activity so radiochemical purity was not definitely established for this compound. The specific activity of the etiocholanolone derivative did not differ significantly from the calculated value.

Table XIII presents the total weight of each 17-ketosteroid

isolated, the final specific activity of each and the minimal per cent conversion of the administered 17α-hydroxypregnenolone-7α-H^3 to dehydroisoandrosterone, androsterone and etiocholanolone in the three experiments. The conversions to the three 17-ketosteroids were 0.90, 0.99 and 0.19 per cent for Experiment I, II and III, respectively. The relative proportions of the three individual conversions making up the total differed for the three experiments.

DISCUSSION

The minimal per cent conversions of the administered 17α-hydroxypregnenolone-7αH^3 to the three urinary 11-desoxy-17-ketosteroids, dehydroisoandrosterone, androsterone and etiocholanolone, in the three experiments reported here are less than 1 per cent (Table XIII). This indicates that the metabolism of the administered compound to the three urinary metabolites is quantitatively only of minor importance in the total metabolism of the C_{21} precursor. Because the transformations are so small, the validity of the final results is very much dependent on the proof of the radiochemical homogeneity of the isolated compounds. Radiochemical homogeneity in these experiments was established by determining the specific activities of the crystals and unfractionated mother liquors resulting from several crystallizations of the free compound and of their derivatives. It is evident from the specific activities of the dehydroisoandrosterone and its derivatives in Experiment I (Table IX) that this method of approach can readily detect radioactive contaminants. In this instance the specific activities of both the acetate and its 5,6α-epoxide were lower than the corresponding calculated values, thus demonstrating that if the specific activity

TABLE XII

SPECIFIC ACTIVITIES OF THE ISOLATED 11-DESOXY-17-KS AND THEIR DERIVATIVES FOR EXPERIMENT III

Steroid	Crystallization	Free Compounds		Derivative	
		Crystals	ML	Crystals	ML
			(cpm/mg)		
Dehydroiso-androsterone	1	266	633		
	2	269	428		
	3	255	369		
	4	273	262		
				3α,17β-diol-3-acetate	
Androsterone	1	654	984	120	105
	2	659	671	164	134
	3			107	94
	4			101	103
	Calculated			122	
				3α,17β-diol-3-acetate-17-benzoate	
Etiocholanolone	1	886	1,345	181	573
	2	832	1,164	167	279
	3	864	862	157	302
	4			152	150
	Calculated			156	

TABLE XIII

FINAL SPECIFIC ACTIVITIES AND AMOUNTS OF 11-DESOXY—17-KETOSTEROIDS ISOLATED AND MINIMAL PERCENTAGE CONVERSION OF INJECTED 17α-HYDROXYPREGNENOLONE TO DEHYDROISOANDROSTERONE, ANDROSTERONE AND ETIOCHOLANOLONE

Experiment	Dehydroisoandrosterone			Androsterone			Etiocholanolone			Total
	Specific Activity cpm/mg.	Weight mg.	Minimal % conversion[1]	Specific Activity cpm/mg.	Weight mg.	Minimal % conversion	Specific Activity cpm/mg.	Weight mg.	Minimal % conversion	Minimal % conversion
I	796	9.4	0.09	2,295	16.2	0.46	2,098	13.5	0.35	0.90
II	195	223.0	0.51	355	66.0	0.28	82	200.0	0.20	0.99
III[2]	273	9.6	0.03	545	9.4	0.06	835	9.8	0.10	0.19

[1] % Conversion = 4(D.Sa + A.Sa + E.Se) x 100/cpm of injected steroid where D, A and E are the daily excretions of dehydroisoandrosterone, androsterone and etiocholanolone, respectively, and Sa, Sa, Se are the specific activities of each.

[2] In Experiment III the amounts of steroid isolated are the weights of each of the three 17-ketosteroids added to the neutral extract.

of the free steroid had been used as a final result it would have been an overestimation. Even after the formation of the second derivative it cannot be stated with certainty that radiochemical homogeneity has been established because its specific activity was lower than the calculated one. If a third derivative had been formed an indication of whether radiochemical homogeneity had been established could have been obtained but this was not possible due to the lack of material at this stage. Lack of steroid also prevented an unequivocal establishment of radiochemical homogeneity of the dehydroisoandrosterone and androsterone in Experiment III. In all of the other metabolites isolated, adequate evidence for radiochemical homogeneity was presented.

Experiments I and II were designed to study the effect of an increased plasma dehydroisoandrosterone level on the in vivo conversion of 17α-hydroxypregnenolone to urinary dehydroisoandrosterone, androsterone and etiocholanolone and to compare the results obtained to those of Solomon et al (119) where a similar study was performed in a patient with metastatic adrenal carcinoma.

In Experiment II it was hoped that by administering dehydroisoandrosterone per os an elevated plasma level of dehydroisoandrosterone would be established at the time of the administration of the labelled 17α-hydroxypregnenolone. In this way, one would be able to determine if an increased blood level could affect the conversion of the injected precursor to urinary 11-desoxy-17-ketosteroids. Such an explanation was possible for the enhanced conversion in the subject with adrenal carcinoma. We administered the 17α-hydroxypregnenolone-7α-H^3 at the

eighth hour, in accordance with our previous findings in trial experiments when 100 mg of dehydroisoandrosterone were administered pas os every hour. Unfortunately, the highest concentration of plasma dehydroisoandrosterone was found at the fourth hour in Experiment II and the levels at the eighth and thirteenth hours were lower although the steroid was still being administered. This decrease in blood concentration cannot be explained with the knowledge available at this time. It is possible that the peak concentration of the plasma dehydroisoandrosterone occurred between the fourth and eighth hours. At the time of the injection the plasma dehydroisoandrosterone was three times the control level.

The minimal per cent conversions of the 17 α-hydroxypregnenolone to the urinary 11-desoxy-17-ketosteroids in Experiments I and II were 0.90 and 0.99 respectively. These two figures are not significantly different and hence the administration of dehydroisoandrosterone in Experiment II did not affect the conversion of the labelled C_{21} steroid to the urinary 17-ketosteroids. Although the total conversion to the three 17-ketosteroids was not affected, the relative conversions to the individual compounds were altered by the administration of dehydroisoandrosterone. There was an increased excretion of dehydroisoandrosterone and a concomitant fall in the formation of androsterone and etiocholanolone (Table XII). In Experiment I the specific activities of the androsterone and etiocholanolone were approximately equal and are two to three times higher than the specific activity of the dehydroisoandrosterone. If the 17α-hydroxypregnenolone was metabolized

only to dehydroisoandrosterone which in turn would label the dehydroisoandrosterone pool, one would expect the specific activities of urinary dehydroisoandrosterone, androsterone and etiocholanolone to be equal in accordance with the finding of Vande Wiele and Lieberman (26, 140). These investigators found that when labelled dehydroisoandrosterone is administered to the normal male, the specific activities of the three 11-desoxy-17-ketosteroids are approximately equal. If a large portion of the labelled dehydroisoandrosterone were metabolized to Δ^4-androstene—3,17-dione immediately after it is formed from 17α-hydroxypregnenolone and before it mixed with the circulating pool of dehydroisoandrosterone then one would expect the specific activity of the urinary dehydroisoandrosterone to be lower. The secretion of dehydroisoandrosterone sulphate by the adrenals could cause the specific activity of the excreted dehydroisoandrosterone to be lower than the values for the excreted androsterone and etiocholanolone. Vande Wiele et al (144) have found in some subjects that urinary dehydroisoandrosterone sulphate has a lower specific activity than the urinary androsterone and etiocholanolone conjugates. This may be due to the failure of dehydroisoandrosterone sulphate to equilibrate with the free steroid. This results in a lowering of the specific activity of the urine dehydroisoandrosterone when it is the pool of free dehydroisoandrosterone that has been labelled. If a portion of the labelled 17α-hydroxypregnenolone was converted to 17α-hydroxyprogesterone the specific activity of the androsterone and etiocholanolone would be higher but one would expect the specific activity of the etiocholanolone to be higher than

that of the androsterone in accordance with the results of Fukushima and his co-workers (116). In Experiment II, the addition of more factors makes it even more difficult to try to explain the differences in specific activities of the urinary 11-desoxy-17-ketosteroids. In this experiment, we administered large quantities of dehydroisoandrosterone which may have overloaded some of the enzyme systems involved in its metabolism, as well as that of the 17∝-hydroxypregnenolone. This might result in a fraction of the steroids being diverted to metabolic pathways not encountered in Experiment I.

That the administered dehydroisoandrosterone did not affect the total conversion of 17∝-hydroxypregnenolone to urinary 17-ketosteroids supports the other possible explanation of the enhanced conversion in the patient with adrenal carcinoma, namely, that the metastatic tissue influenced the conversion. This possible explanation is also supported by the fact that the metastatic tissue excised from the patient was capable of transforming cholesterol and 17∝-hydroxypregnenolone to dehydroisoandrosterone (204).

Experiment III demonstrates that the presence of endocrine tissue may be in part responsible for the in vivo conversion of 17∝-hydroxypregnenolone to urinary dehydroisoandrosterone, androsterone and etiocholanolone. In this study performed on a subject who had been adrenalectomized, ovariectomized and hypophysectomized, the per cent conversions were considerably smaller than in the normal subject of Experiment I. In this experiment the conversion to etiocholanolone was higher than the transformations to the other two steroids. This could be explained if the 17∝-hydroxypregnenolone was

converted to 17α-hydroxyprogesterone. The metabolism of this C_{21} steroid favours the formation of etiocholanolone over that of androsterone.

Recently, Fukushima et al (205) have reported on the metabolism of 17α-hydroxypregnenolone and 17α-hydroxyprogesterone in a subject with adrenal carcinoma and on the metabolism of 17α-hydroxypregnenolone in a normal subject. In the cancer patient, the conversions of the administered 17α-hydroxypregnenolone to urinary dehydroisoandrosterone, androsterone and etiocholanolone were 1.7, 0.5 and 1.1 per cent respectively. The principal metabolites were C_{21} steroids (e.g. pregnanetriol). The 17-ketosteroids were not studied in the normal subject. Roberts et al (120) found that the conversions of 17α-hydroxypregnenolone to dehydroisoandrosterone, androsterone and etiocholanolone by a patient with an adrenal adenoma were 0.95, 0.24 and 2.8 per cent respectively. Our studies and those of other workers indicate that in pathological conditions where abnormally large amounts of steroids are being produced, the pattern of metabolism of steroids is altered. MacDonald et al (143) measured the conversions of labelled dehydroisoandrosterone to urinary dehydroisoandrosterone, androsterone and etiocholanolone under several conditions in the same subject. When the secretion of dehydroisoandrosterone was altered by the administration of dexamethazane or ACTH, the per cent of the administered dehydroisoandrosterone excreted as androsterone and etiocholanolone did not change. However, the fraction of the administered radioactivity excreted as dehydroisoandrosterone varied with its rate of secretion and large

amounts were found in the urine when the secretion rate was enhanced.

In conclusion, it would appear that the peripheral conversion of 17α-hydroxypregnenolone to 17-ketosteroids is quantitatively not important except in certain cases of adrenal carcinoma with metastases. However, it is probably the important adrenal precursor of dehydroisoandrosterone.

PART II.

A STUDY ON THE ORIGIN OF PREGNANETRIOL, PREGNENETRIOL and 17α-HYDROXY-PREGNANOLONE IN A CASE OF BILATERAL ADRENAL HYPERPLASIA.

INTRODUCTION

Pregnane-3α,17α,20α-triol, the 3,17,20-trihydroxypregnane most commonly found in urine (referred to as pregnanetriol in this thesis), was first isolated from the urine of two women with adrenogenital syndrome by Butler and Marrian (206) in 1937. It was observed that following the removal of an enlarged adrenal gland from one of these patients, pregnanetriol was no longer found in the urine. In addition, it was not found in the urine of normal human males and females or in the urine of pregnant females. In 1938 these same authors (207) reported the isolation of pregnanetriol from an extract of unhydrolysed urine obtained from a woman with an adrenal tumour.

In 1945, Mason and Kepler (10) reported on the steroids isolated from the urine of patients with adrenocortical tumours and adrenocortical hyperplasia. They found pregnanetriol in the urine of three of the four cases with adrenal hyperplasia and in the urine of only one of the six subjects with adrenal tumours. Using the method of Venning (49) for isolating conjugates, they found both pregnanediol and pregnanetriol following hydrolysis of the purified glucosiduronidate fraction. Miller and Dorfman (208) also found pregnanetriol in the urine of a subject with (probable) adrenocortical hyperplasia.

The first report of the isolation of pregnanetriol from the urine of normal subjects was that of Cox and Marrian (209) who isolated pregnanetriol from extracts of urine obtained from normal men. Further evidence that pregnanetriol is excreted as the glucosiduronidate was

the finding that the steroid was extractable from the urine only after β-glucuronidase hydrolysis and that if the enzyme was boiled before use or saccharate (a known β-glucuronidase inhibitor) was added, the steroid was not found in the urine extract.

Bongiovanni (210) reported in 1953 that in many cases of adrenogenital syndrome with adrenal hyperplasia the large increase in the urinary pregnanediol reported by other workers appeared to be due to the presence of large quantities of pregnanetriol which in most of the earlier methods was not separated from pregnanediol. It was concluded that urinary pregnanetriol was characteristic of such patients. Further proof of its adrenal origin was the fact that cortisone administration inhibited the excretion of pregnanetriol. Adrenocorticotrophin, when administered to a subject with adrenal hyperplasia, caused an increase in urinary pregnanetriol and pregnanediol and plasma hydrocortisone. No such changes in pregnanediol and pregnanetriol excretion were obtained when the same dose was given to two normal children of the same age group.

Several isomers of pregnanetriol have been found in urine. In 1956, deCourcy (211) reported the presence of pregnane-3α,17α, 20β-triol in the urine of a subject with adrenocortical hyperplasia. Fukushima and Gallagher (212) isolated allopregnane-3α,17α,20α-triol and obtained very good evidence for the presence of allopregnane-3α,17α, 20β-triol in the urine of a subject with congenital adrenal hyperplasia. The urinary level of pregnanetriol for this patient was 65 mg/24 hours.

The compound 17α-hydroxypregnanolone which differs from pregnanetriol only in that the oxygen function on carbon-20 is a ketone, was first isolated from urine in 1945 by Lieberman and Dobriner (213). They isolated this steroid from the urine of a woman with adrenocortical hyperplasia, a cryptorchid male, a woman with an adrenal tumour and an eunuchoid male being given testosterone by injection. Mason and Strickler (214) isolated 17α-hydroxypregnanolone from the urine of a female pseudohermaphrodite. The authors isolated the 17α-hydroxypregnanolone from a purified glucosiduronidate fraction of the urine, indicating that 17α-hydroxypregnanolone is also excreted in the urine as a glucosiduronidate.

The unsaturated triol, Δ^5-pregnene-3β,17α,20α-triol (pregnenetriol was first isolated by Hirschmann from the urine of a boy with adrenocortical carcinoma (185). This boy, as discussed in Part I, was excreting large quantities of steroids with the 3β-hydroxy-Δ^5 structure. Pregnenetriol was first isolated from the urine of normal subjects by Fotherby (188) who suggested that it might be a precursor of dehydroisoandrosterone. In 1959, Okada et al (22) reported the isolation of pregnenetriol in considerable quantities from the urine of a subject with adrenocortical carcinoma.

Urinary Excretion of Pregnanetriol, Pregnenetriol and 17α-Hydroxypregnanolone

One of the first methods for the determination of urinary pregnanetriol was that of Bongiovanni and Clayton (215). Their method involved separation of the pregnanetriol from pregnanediol by chroma-

tography on an alumina column. The pregnanetriol was quantitated by measurement of the colour intensity preduced with concentrated sulphuric acid. Using this method they found that the urinary excretion of pregnanetriol for normal adults ranged from 0.2 to 1.8 mg/24 hours. An earlier method by Cox (216) which measured all C_{21} steroids with a 17,20-dihydroxy-20-methyl side chain gave a range of 0.1 to 0.5 mg/24 hours for 7 normal males. However, this latter method did not involve hydrolysis and hence only unconjugated steroids were measured. It is well known that the larger fraction of the triols are excreted as conjugates and Cox stated that the levels would have been higher if the urine had been treated with β-glucuronidase prior to extraction.

Bongiovanni and Eberlein (217) found that the urinary excretion of pregnanetriol varied with age. The urine levels for normal children less than six years of age ranged from 0 - 0.2 mg/24 hours (average 0.02 mg), those for children between the ages of 7 and 16 ranged from 0.3 to 1.1 mg/24 hours (average 0.6 mg) and for adult subjects the range was from 0.2 to 3.5 mg (average 1.53 mg).

Cox (218) analysed the urine of two normal subjects for four of the triols and obtained levels of 0.4 and 0.5 mg/24 hours for pregnanetriol, 0.05 and 0.03 mg/24 hours for pregnane-3α,17α,20β-triol, 0.07 and 0.05 mg/24 hours for allopregnane-3α,17α,20α-triol and 0.1 and 0.08 mg/24 hours for pregnenetriol. Of the saturated triols, pregnanetriol is quantitatively the most important. Fotherby and Love (219) found that urinary pregnanetriol of normal adults decreases with age. The mean level for female subjects who were 34 to 38 weeks pregnant was higher than that of the normal non-pregnant females

(2.0 versus 0.9 mg/24 hours). The levels of both pregnanetriol and pregnenetriol were found to be significantly below normal for subjects with various types of hypertension (220).

Origin of Urinary Pregnanetriol, 17α-Hydroxypregnanolone and Pregnenetriol

There is published evidence which suggests that the adrenals, ovaries and testes are all sources of pregnanetriol, 17α-hydroxypregnanolone and pregnenetriol. Much of the work with regard to the origin of these compounds has been in relation to adrenocortical hyperplasia, especially that of the congenital variety. As mentioned, pregnanetriol was first isolated from the urine of two women (206) who had the adrenogenital syndrome. It had also been concluded by Bongiovanni (210) in 1953 that elevation of pregnanetriol was characteristic of adrenocortical hyperplasia.

In the series of patients with the adrenogenital syndrome studied by Bongiovanni et al (215, 217, 221) the urinary levels of pregnanetriol were all above normal. The level of pregnanetriol excretion was decreased to low normal levels by the administration of cortisone, which is known to lead to decreases in adrenal steroid biosynthesis by suppressing ACTH release by the pituitary. Childs et al (222) found that the level of urinary pregnanetriol was increased by the administration of ACTH to a greater extent in subjects with virilising adrenocortical hyperplasia than in normals.

The excretion of urinary steroid metabolites by a boy with adrenocortical hyperplasia was studied by Bush et al (223). The urinary excretion of pregnanetriol was 41 mg/24 hours. Also found were two

compounds not normally detected in the urine of boys, 17α-hydroxypregnanolone and 11-ketopregnanetriol. The administration of ACTH led to an increase in the urinary level of 17α-hydroxypregnanolone but not in that of pregnanetriol. Treatment with cortisone resulted in decreases in the urinary levels of both pregnanetriol and 17α-hydroxypregnanolone to less than 1 mg/24 hours. These studies indicate that the adrenal is capable of producing large quantities of the precursors of the urinary metabolites. The level of urinary pregnanetriol was found by Cox and Finkelstein (224) to be high for subjects with either adrenocortical hyperplasia or adrenal tumours. In a later report (225) the level of urinary pregnanetriol was found to be normal in some cases of adrenocortical carcinoma. Cox (218) also found that the levels of pregnane-3α,17α,20β-triol, allopregnane-3α,17α,20α-triol and pregnenetriol were above normal in two subjects with adrenocortical hyperplasia and one subject with adrenocortical carcinoma. The levels of pregnenetriol have been found to be above normal for most subjects with adrenocortical carcinoma (226).

Bongiovanni et al (227) measured the urinary levels of both pregnanetriol and 17α-hydroxypregnanolone and found them to be significantly elevated in children with adrenocortical hyperplasia and slightly elevated in a subject with a virilizing adrenal tumour.

All of these studies in subjects with adrenocortical hyperplasia implicate the adrenals as being the origin of a large fraction of the urinary pregnanetriol in such subjects and suggests that they may be the origin of the small quantities excreted by normals. This suggestion is supported by the studies on adrenal steroid biosynthesis and the find-

ing of the presumed precursor, 17α-hydroxyprogesterone, in adrenal venous blood. These studies have been discussed in Part I. The reason for the increased excretion of pregnanetriol by subjects with congenital adrenal hyperplasia is believed to be a defect in hydroxylation of the steroid intermediates at the C_{21} position with the result that 17α-hydroxyprogesterone is secreted in increased quantities. Since the adrenal is unable to synthesize hydrocortisone, the pituitary reacts by secreting greater quantities of ACTH and consequently stimulating the adrenals to produce larger quantities of the biosynthetic intermediates. In support of this hypothesis, Bongiovanni (228) found that adrenals from such a subject could not hydroxylate 17α-hydroxyprogesterone at Carbon 21 while they could hydroxylate 11-desoxyhydrocortisone to form hydrocortisone.

Recently, Bongiovanni (229, 230) has described a new variant of congenital adrenal hyperplasia in which there appears to be a defect in the 3β-hydroxydehydrogenase reaction with the result that there is an excess excretion of compounds containing the 3β-hydroxyl-Δ^5 structure. These include pregnenetriol, Δ^5-pregnenediol, 17α-hydroxypregnenolone and dehydroisoandrosterone. No pregnanetriol is excreted by the majority of these subjects. These results indicate that the adrenals can also be the source of urinary pregnenetriol. Fukushima and his co-workers (231) studied the effect of an inhibitor of 11β-hydroxylase (SU-4885) on the urinary excretion of steroids by a patient with adrenocortical carcinoma and found more than a two-fold increase in the level of Δ^5-pregnene-3β,17α,20α-triol and a small increase in the level of urinary Δ^5-pregnene-3β,17α,20β-triol. The increase in

pregnanetriol was insignificant.

In recent years there have been a number of reports in the literature which indicate that the ovary is capable of secreting precursors of both urinary pregnanetriol and pregnenetriol. There is evidence which indicates that pregnanetriol is derived from an ovarian precursor under normal conditions while increased levels of urinary pregnenetriol have been found in association with the Stein-Leventhal syndrome.

Picket et al (232) reported in 1959 that the urinary excretion of pregnanetriol closely followed the cyclical variation in pregnanediol excretion in a normal female subject with the peak levels occurring during the luteal phase of the menstrual cycle. The changes in steroid excretion during the menstrual cycle for normal females have always been attributed to changes in ovarian activity. When the urinary pregnanetriol levels in an adrenalectomized female subject were measured, an increase in excretion was observed during the second half of the menstrual cycle.

In 1960, Fotherby (233) reported on the excretion of various steroids by normal females during the menstrual cycle and found no variations in the urinary levels of 17-ketosteroids, 17-hydroxycorticoids and dehydroisoandrosterone. Cyclic changes were found for pregnanetriol and pregnanediol. Picket et al (232) had indicated that the changes occur simultaneously. Fotherby (233) found that the changes in the excretion of the pregnanetriol preceded those of the pregnanediol. In later reports, Fotherby (234, 235) also showed that progestational steroids inhibit the rise in the excretions of both pregnanetriol and pregnanediol during the menstrual cycle. He concluded that the

pregnanetriol was probably ovarian in origin.

That the increase during the luteal phase of the menstrual cycle was really due to pregnanetriol and not to similar chromogens was confirmed by Picket and Kellie (236). More recent studies by Picket and Somerville (237) confirm the results of Fotherby that the peak in pregnanetriol excretion occurs prior to that of pregnanediol. Burger and Somerville (238) obtained a four-fold increase in pregnanetriol excretion after administration of human pituitary gonadotrophin to an adrenalectomized subject. No increase was obtained following ACTH administration. These data lend further support to the idea of an ovarian source of pregnanetriol.

The urinary excretion of pregnenetriol was found to be elevated in patients with the Stein-Leventhal syndrome both before and after wedge resection of the ovaries (239). Mahesh and Greenblatt (240) studied a patient with the Stein-Leventhal syndrome and noted a ten-fold increase in the urinary excretion of both pregnanetriol and pregnenetriol following the administration of FSH while only a four-fold increase was observed following ACTH injections. The urinary 17-hydrocorticoids in this patient were not elevated following the FSH administration, indicating that it was stimulating the ovaries and not the adrenals. Stern and Barwell (241) reported that ten of eleven patients with the Stein-Leventhal syndrome excreted pregnenetriol at levels greater than normal subjects. These findings agree with those of Mahesh and Greenblatt (78) who detected 17α-hydroxypregnenolone (which is believed to be the precursor of pregnenetriol) in ovarian

sections obtained from patients with the Stein-Leventhal syndrome.

Administration of HCG increased the urinary excretion of pregnanetriol by a series of nine men with functioning testes (242). An increase was observed in a similar study on a male with Addison's disease but not in a male eunochoid. This indicates that the testes are capable of secreting pregnanetriol precursors. The level of urinary pregnanetriol was quite high in a subject with testicular feminizing syndrome (243) and was reduced to below normal levels following the removal of cryptorchid testes.

The Metabolic Conversion of C_{21} Steroids to Pregnanetriol, Pregnenetriol and 17α- Hydroxypregnanolone.

The results of studies in subjects with congenital adrenal hyperplasia have led to the conclusion that the high urinary excretion of pregnanetriol is due to an excessive secretion of 17α-hydroxyprogesterone. To check this hypothesis a number of workers have studied the metabolism of 17α-hydroxyprogesterone administered to human subjects.

Bongiovanni et al (221) observed an increase in urinary pregnanetriol following the intramuscular administration of 17α-hydroxyprogesterone to subjects with the adrenogenital syndrome but not with normal subjects. Langecker and Prescher (244) obtained increases in the urinary pregnanetriol levels following the intramuscular administration of 17α-hydroxyprogesterone and 17α-hydroxyprogesterone caproate to a normal male. Langecker (245) was also able to isolate 17α-hydroxypregnanolone as well as pregnanetriol from the urine of a normal male following 17α-hydroxyprogesterone administration. Similar results were

obtained by Jailer et al (110).

Ungar et al (246) reported that administration of 17α,21-dihydroxy-5β-pregnane-3,20-dione, a possible metabolite of 11-desoxycortisol, gave an increase in urinary pregnanetriol following its administration to a human subject. Such a conversion would involve the removal of the C_{21} hydroxyl group and the results of Fukushima et al (247) do not support such a conversion.

The first quantitative experiments concerned with precursors of urinary pregnanetriol were those of Fotherby and Love (248). These investigators reported that intravenously administered 17α-hydroxyprogesterone, 17α-hydroxypregnenolone and 11-desoxyhydrocortisone were converted to urinary pregnanetriol in yields of 35, 8 and 0 per cent respectively by both normal and adrenalectomized subjects. They also found that 66 per cent of an administered dose of pregnanetriol was excreted unchanged.

Axelrod and Goldzieher (249) administered 17α-hydroxyprogesterone orally to four subjects and identified pregnane-3α,17α,20α-triol, pregnane-3α,17α,20β-triol, allopregnane-3α,17α,20α-triol, allopregnane-3α,17α,20β-triol, pregnane-3α,17α-diol-20-one, allopregnane-3α,17α-diol-20-one and allopregnane-3β,17α-diol-20-one as urinary metabolites. Quantitatively the two major metabolites were pregnane-3α,17α,20α-triol (pregnanetriol) and pregnane-3α,17α-diol-20-one (17α-hydroxypregnanolone). Of an oral dose of 17α-hydroxypregesterone administered to a patient with congenital adrenal hyperplasia, 12 per cent of the dose was excreted as pregnanetriol and 6 per cent as 17α-hydroxypregnanolone (250).

In 1961, Roberts et al (120) reported on the conversions of labelled 17α-hydroxypregnenolone to urinary pregnanetriol and pregnenetriol in a subject with a virilizing adenoma of the adrenal. The minimal conversions to pregnanetriol and pregnenetriol were 6.4 and 22.0 per cent respectively. However, the specific activities of the two urinary metabolites did not differ significantly. This indicated that the principal precursor of urinary pregnanetriol in this subject was 17α-hydroxypregnenolone since the specific activity of the pregnanetriol would have been lower than that of pregnenetriol if 17α-hydroxyprogesterone were being secreted in significant quantities. It is important to note that this subject was excreting relatively large quantities of compounds with the 3β-hydroxy-Δ^5 structure. The urinary excretions of pregnenetriol and pregnanetriol were 13 and 4 mg/24 hours respectively.

Fukushima and his co-workers have reported several studies on the origin of urinary pregnanetriol. In 1961, they (116) reported on the metabolism of labelled 17α-hydroxyprogesterone by a normal subject and in an untreated subject with congenital adrenal hyperplasia. They found radioactive pregnanetriol, pregnane-3α,17α,20β-triol, allopregnane-3α,17α,20α-triol and 17α-hydroxypregnanolone in the urine. Pregnanetriol and 17α-hydroxypregnanolone were the major metabolites. In the patient with congenital adrenal hyperplasia the specific activities of both the pregnanetriol and 17α-hydroxypregnanolone for each of the three days following the administration of the labelled precursor were measured. From the changes of these specific activities it was concluded that 17α-hydroxypregnanolone is the precursor of pregnanetriol. A

larger fraction of the injected dose was excreted as pregnanetriol than as 17α-hydroxypregnanolone in this patient. However, the cumulative specific activities of these two steroids over the three days of the study were the same, suggesting a common precursor. A similar pattern of excretion of radioactivity was found in the urine of a normal subject. However, the fraction of radioactivity appearing in each of these two metabolites was the same in this study.

In a separate study by Fukushima et al (117) on a patient with adrenal carcinoma who was excreting approximately 120 mg of pregnanetriol per day it was found following the administration of labelled 17α-hydroxyprogesterone that the specific activity of the urinary 17α-hydroxypregnanolone was more than 10 times greater than the specific activity of the pregnanetriol. The conversions to pregnanetriol and 17α-hydroxypregnanolone were 10.6 per cent and 16.5 per cent, respectively, and the daily excretion of 17α-hydroxypregnanolone was 8.7 mg. Because of the large difference between the specific activities of the two metabolites it was concluded that there was at least one other precursor of the urinary pregnanetriol. It was calculated that only about 5 per cent of the urinary pregnanetriol came from endogenous 17α—hydroxyprogesterone so if there were only one other precursor, it would be the more important one. It was suggested that 17α-hydroxypregnenolone might be this precursor as the urinary excretion of one of its metabolites, pregnenetriol, was 40 mg per day, indicating that it was being produced in large amounts.

In a more recent study, Fukushima et al (205) have measured

the transformations of 17α-hydroxyprogesterone-C^{14} and 17α-hydroxypregnenolone-H^3 administered simultaneously to urinary pregnanetriol, pregnenetriol and 17α-hydroxypregnanolone in a patient with adrenal carcinoma. The conversions of 17α-hydroxyprogesterone to pregnanetriol and 17α-hydroxypregnanolone were 8.8 and 7.9 per cent, respectively. The conversions of 17α—hydroxypregnenolone to pregnanetriol, 17α-hydroxypregnanolone and pregnanetriol were 6.6, 0.6 and 11.2 per cent, respectively. From the specific activities it can be concluded that 17α-hydroxyprogesterone in this subject was a more important precursor of 17α-hydroxypregnanolone than of pregnanetriol. The specific activities of the pregnanetriol and pregnenetriol released by the β-glucuronidase hydrolysis differed by approximately 15 per cent with regard to its tritium content. However, the pregnenetriol released by cold acid hydrolysis which cleaves the steroid sulphates, had a specific activity much less than the pregnenetriol released by β-glucuronidase (2,200 cpm/mg versus 3,300 cpm/mg). It was suggested that this difference could be attributed to the secretion of 17α-hydroxypregnenolone sulphate which would not mix with the injected hormone and would thus lower the specific activities of the sulphated urinary metabolites. It should be noted that the daily excretion of pregnenetriol (43.2 mg) indicates an excessive secretion of a Δ^5-compound since pregnenetriol would probably have such a precursor. Pregnenetriol has not been found in adrenal venous blood. The metabolism of 17α-hydroxypregnenolone was also studied in a normal subject. The conversions to pregnanetriol,17α-hydroxypregnanolone and pregnenetriol were 5.1, 1.3 and 8.8 per cent, respectively.

Recently, a study by Bradlow et al (251) has indicated that the peripheral conversions of administered steroids can be significantly affected by drugs which inhibit steroid production. In this study O,P'-DDD, which suppresses steroid production, diminished the conversion of 17 α-hydroxypregnenolone to pregnanetriol while the formation of pregnenetriol was unchanged.

An in vitro study on the metabolism of 17α-hydroxypregnenolone which yielded interesting results was that of Thomas et al (252). These authors found that rabbit skeletal muscle strips converted labelled 17α-hydroxypregnenolone to the 20α- and 20β- isomers of pregnenetriol in yields of 16.2 and 2.2 per cent respectively. These reactions, which appeared to be enzymatic, indicated that tissues other than liver may be important in the formation of these compounds from the secreted precursors.

The results of a number of the investigations discussed above indicate that pregnanetriol and 17α-hydroxypregnanolone are excreted almost exclusively as the glucosiduronidates. However, it appears that pregnenetriol is excreted in part as the glucosiduronidate and part as the sulphate.

The subject of this study was a female with Cushing's syndrome due to bilateral adrenal hyperplasia. Studies were performed before and after bilateral adrenalectomy. The aim of each study was to measure the conversions of simultaneously administered 17α-hydroxypregnenolone-7α-H^3 and 17α-hydroxyprogesterone-4-C^{14} to urinary pregnanetriol, 17α-hydroxypregnanolone and pregnenetriol and to determine the effect of the presence of the

adrenals or their secretory products on these transformations.

EXPERIMENTAL

METHODS

A number of the experimental procedures used in this section have been described in Part I. These include the preparation of samples for counting, infra red analysis, hydrolysis of the urinary steroid conjugates, the initial silica gel chromatography of the urine extracts, acetylations and sodium borohydride reductions. These procedures will not be described here.

CHROMATOGRAPHIC PROCEDURES

Following the initial silica gel chromatography, the combined column fractions were further purified by thin layer chromatography (T.L.C.). The plates for T.L.C. were prepared from a slurry of 50 gm of silica gel G and 100 ml of distilled water and spread to a thickness of 1 mm on 4 glass plates, 8 in. x 8 in. The solvent system used in these studies was ethyl acetate : n-hexane (4:1). Small quantities of several steroids were run as standards on one side of the plate. After the chromatogram had been developed and the plates dried for a minimum of 30 minutes, the standards were visualized by spraying with phosphomolybdic acid (10 per cent solution in absolute ethanol) and heating the plate in an oven for several minutes at 100 — 120°C. This method of detecting steroids was originally devised by Kritchevsky and Kirk (253) for visualizing steroids on paper chromatograms.

In several instances the pregnenetriol fractions from the thin layer chromatograms were chromatographed on a silica gel partition type column prepared according to Katzenellenbogen et al (254). The silica gel used was obtained from the Davison Chemical Company. For preparation of the columns, 4 ml of ethanol per 10 gm of silica gel were thoroughly mixed with the support and a slurry was made in methylene chloride containing 2 per cent ethanol. The slurry was transferred to the column, which contained 2 per cent ethanol in methylene chloride, with tapping of the column to release air bubbles. The sample was applied to the column by dissolving it in a minimal amount of ethanol and then adding a corresponding weight of silica gel (1 gm per 0.4 ml). The mixture was stirred and a slurry was prepared in 2 per cent ethanol in methylene chloride and packed on top of the column. The column was developed first with 2 per cent ethanol in methylene chloride and then with 3, 4, 5, 6, 8, and 10 per cent ethanol in methylene chloride in order of increasing ethanol concentration. The column was developed at a maximum flow rate of 10 ml per 30 minutes. The pregnenetriols were usually eluted by 4 per cent ethanol in methylene chloride.

In the study performed prior to bilateral adrenalectomy, the pregnanetriol was separated from the corticoid metabolites, which were being excreted at high levels, by a partition column using Celite as the support. The Celite was prepared by first washing it with concentrated hydrochloric acid and then continuously with tap water for 48 hours. This was followed by several washes with distilled water and then methanol. The Celite was then dried overnight in an oven at 80 -

90°C. The solvent system used for the column was that suggested by Schneider (255) who had developed these systems for separating compounds differing only in the configuration of the hydroxyl group at carbon-20 by paper chromatography (256). Two solvent systems were required for the column: System A: n-heptane (100), isopropyl ether (100), methanol (150) and water (50), plus 15 gm of boric acid which was dissolved in the methanol and water prior to addition of the other two solvents. System B: Same as System A but lacking the boric acid. For preparation of the column the Celite was suspended in the upper phase of System B and the desired amount of the lower phase of System A (1 ml per 2 gm of Celite) was added and the mixture was thoroughly stirred. The column was packed and developed in the upper phase of System B.

Several paper chromatographic systems not used in Part I were employed during these experiments. The 17α-hydroxy pregnanolone was purified by chromatography on paper in the toluene : propylene glycol system (257). The pregnenetriols were purified in the Bush type system of Wilson et al (191), ligroin : toluene : methanol : water (50, 50, 70, 30). The corresponding standards were located by dipping in a solution of phosphomolybdic acid in ethanol (10% w/v), blotting and heating in an oven up to 60°C. Radioactive areas on the peper chromatograms were located with chromatogram scanner (Vanguard model 880). A 3 cm strip from the middle of the paper was used for scanning.

Sodium Bismuthate Oxidations: In the preparation of derivatives of pregnanetriol and 17α-hydroxypregnanolone, the side chain was cleaved

to form the corresponding 17-ketosteroid. The reaction was performed according to the method described by Appleby et al (258). A solution of 20 mg of steroid per ml of aqueous tert-butanol : water (80:20) was prepared. In some instances it was found that the steroid would not dissolve in such a small volume of solvent, so larger volumes were used (e.g. up to 4 ml per 20 mg of steroid), without affecting the results of the reaction. To the steroid solution were added 20 ml of 50% acetic acid and 1 gm of sodium bismuthate. The mixture was shaken in the dark for 1 hour and then excess reagent was reduced with 4 ml of 10 per cent sodium thiosulphate. Twenty ml of 3 N NaOH were added to the reaction mixture and it was extracted with ethyl acetate, (3 x 75 ml). The combined ethyl acetate extracts were washed with 3 N NaOH (3 x 20 ml), with distilled water until neutral, dried over sodium sulphate and evaporated to dryness on the flash evaporator. With larger amounts of steroid, all quantities of reagents were increased proportionately.

Preparation of Pregnenetriols: Because Δ^5-pregnene-3β,17α, 20α-triol and Δ^5-pregnene-3β,17α,20β-triol were not commercially available in the quantities required for use as carriers and standards, they were prepared chemically.

The 20α isomer was prepared from 17α-hydroxypregnenolone acetate by reduction with lithium aluminum hydride as described by Hirschmann (259). A total of 200 mg of 17α-hydroxypregnenolone acetate was dissolved in 157 ml of anhydrous ether. This solution was added to a mixture of 420 mg of $LiAlH_4$ in 18.8 ml of ether in a 300 ml 3-neck flask. The steroid solution was added slowly through a dropping funnel while a reflux condenser was connected to a second neck. The mixture

was continuously stirred with a magnetic stirrer. After all the steroid was added, stirring was continued for 30 minutes. The excess lithium aluminum hydride was destroyed by adding moist ether followed by distilled water. The resulting mixture was then partitioned between 300 ml of 0.1 N HCl and 500 ml of ether. Then the aqueous fraction was extracted with a further 200 ml of ether followed by 200 ml of ethyl acetate. The combined extracts were washed with 1 N NaOH (2 x 30 ml) and distilled H_2O until neutral. The extracts were then dried over anhydrous sodium sulphate and evaporated to dryness on the flash evaporator. The dried extract weighed 187.5 mg. Chromatography of a 1/100 aliquot of the extract by T.L.C. in the system ethyl acetate : n-hexane (4:1) indicated the presence of both the 20α and 20β isomers of pregnenetriol with the former predominating, plus a small quantity of unreacted 17α-hydroxypregnenolone. The remainder of the extract was divided into 16 parts and each was chromatographed on thin layer plates in the above system. The areas corresponding to the 20α isomer of pregnenetriol on the plates were combined and eluted.

The crude pregnenetriol fraction (weight 111 mg) was then chromatographed on the partition type silica gel column described above. Forty gm of silica gel were prepared and packed in a column containing 3 per cent ethanol in methylene chloride and developed with 3 and then 4 per cent ethanol in methylene chloride. The column fractions were taken to dryness under vacuum. There was crystalline material in every fraction from 6 to 100 with an apparent break at fractions 12 - 13. Infra red analysis of aliquots of several fractions showed that fractions 6 — 12 contained Δ^5-pregnene-3β,17α,20β-triol, while fractions 14 - 110 con-

tained Δ^5-pregnene-3β, 17α,20α-triol. Fractions 14 — 110 (84.8 mg) were combined, filtered and crystallized three times from methanol-ether. The melting point of the final crystals was 221.5 - 224°C (Corr.). This agreed with the reported value of 221 — 224°C (259). The infrared spectrum was identical to that of the standard Δ^5-pregnene-3β,17α,20α-triol.

To prepare pregnene-3β,17α,20β-triol in considerable quantity, 200 mg of 17α-hydroxypregnenolone was reduced with sodium borohydride as described in Part I. The resulting extract was divided into 20 parts for thin layer chromatography in the system ethyl acetate : n-hexane (4:1). The areas corresponding to the 20β isomer of pregnenetriol were combined, eluted and chromatographed on a silica gel column, prepared in methylene chloride and developed with mixtures of ethanol and methylene chloride (1:99 to 20:80) in a step-wise manner. The Δ^5-pregnene-3β,17α,20β-triol was eluted by 4 - 8 per cent ethanol in methylene chloride. It was then crystallized three times from methanol-ether. The melting point of the final crystals was 226 — 228°C (Corr.). The melting point listed in the literature for Δ^5-pregnene-3β,17α,20β-triol is 224 - 228°C (259). The infrared spectrum was identical with the spectrum of the standard compound.

CHECK OF PURITY OF 17α-HYDROXYPREGNENOLONE-7α-H^3 and 17α-HYDROXYPROGESTERONE—4-C^{14}

The 17α-hydroxypregnenolone-7α-H^3 used in these experiments was the same lot as that used for the experiments in Part I. The data on its purity was given in that section.

The 17α-hydroxyprogesterone-4-C^{14} (0.043 uc/ug) was obtained from New England Nuclear Corporation. In order to test its purity, 7.41×10^5 cpm were mixed with 103.7 mg of non-radioactive carrier. The resulting mixture was then crystallized three times. After each crystallization, approximately 1 mg of the crystals were removed and accurately weighed before transfer to a vial for counting. The mother liquors were accurately weighed, dissolved in methanol and a known aliquot was removed for counting. The specific activities of the crystals and mother liquors after each crystallization were determined.

Subject: The subject used for the two experiments in this study was a 19-year-old female who was a patient of Dr. E.A.H. Sims at the University of Vermont in Burlington. The clinical diagnosis on this patient was one of Cushing's syndrome. She was obese and hirsute and she had striae and a dorsal fat pad. Her plasma electrolytes were normal. The results of urinary steroid analyses preformed in the Endocrinology Laboratory of the Royal Victoria Hospital, Montreal, are presented in Table XIV. The patient underwent bilateral adrenalectomy. At operation the left adrenal had a moderately thick cortex and small areas of medullary tissue. The zona glomerulosa was thin but the zona fasiculata and reticularis were prominent. The right adrenal had a thinner cortex. The glands were normal morphologically and were in the upper limits of normal size. The left adrenal weighed 8 gms and the right, 6.8 grams. The pathology report stated that the histological appearance was consistent with a diagnosis of Cushing's syndrome. Following the operation the patient was maintained on 37.5 mg of cor-

tisone per 24 hours. Four months later the patient's obesity and buffalo hump had markedly decreased. Urinary steroid analyses results on a specimen collected 14 days after the operation are shown in Table XIV. The patient was on cortisone therapy during the collection.

EXPERIMENTAL DESIGN:

Experiment IV: Seven days prior to operation, a mixture of 7.88 x 10^6 cpm of 17α-hydroxypregnenolone-7α-H^3 and 1.06 x 10^6 cpm of 17α-hydroxyprogesterone-4-C^{14} was administered intravenously as described in Part I.

Experiment V: Four months following the operation the patient received an injection containing 7.71 x 10^6 cpm of 17α-hydroxypregnenolone-7α-H^3 and 1.04 x 10^6 cpm of 17α-hydroxyprogesterone-4-C^{14} in the same manner.

Urine was collected for 4 days following the injections. The steroid conjugates in the individual urines were hydrolysed with β-glucuronidase and by solvolysis as described previously. For each study the four extracts obtained after β-glucuronidase treatment and after solvolysis were combined. It was decided to look for 17α-hydroxypregnanolone, pregnanetriol, Δ^5-pregnene-3β,17α,20α-triol and Δ^5-pregnene-3β,17α,20β-triol in the glucosiduronidate extracts and Δ^5-pregnene-3β,17α,20α-triol and Δ^5-pregnene-3β,17α,20β-triol in the sulphate extracts. To facilitate isolation of the steroids from the neutral extracts in Experiment V, small quantities of the anticipated metabolites were added as carrier before chromatography. The following steroids were added to the extract obtained after β-glucuronidase treatment -

TABLE XIV

URINARY STEROID VALUES FOR SUBJECT BEFORE AND AFTER BILATERAL ADRENALECTOMY

	Pre-operative (mg/24 hours)	Post-operative* (mg/24 hours)	Normal Range (mg/24 hours)
Pregnanediol	4.4	0.3	
17-Hydroxycorticoids	17.1	9.2	2 - 6
17-Ketosteroids	22.9	3.3	6 - 16
17-Ketogenic Steroids	65.5	48.2	6 - 12
Pregnanetriol	1.3	0	0.7 - 2.5

* Maintained on 37.5 mg of cortisone per day and 0.1 mg of 9α-fluorohydrocortisone per day.

6.59 mg pregnanetriol, 8.31 mg 17α-hydroxypregnanolone, 2.00 mg Δ^5-pregnene-3β,17α,20α-triol and 3.50 mg Δ^5-pregnene-3β,17α,20β-triol. To the combined extracts obtained after solvolysis, were added 5.71 mg Δ^5-pregnene-3β,17α,20α-triol and 4.32 mg Δ^5-pregnene-3β,17α,20β-triol. All four extracts were then chromatographed on silica gel columns as described in Part I. The elution patterns of radioactivity for each of the two columns for Experiments IV and V are shown in Figures VI and VII, respectively.

In Experiment IV, peaks B and C from the column of the glucosiduronidate extract were combined and chromatographed on T.L.C. in order to isolate 17α-hydroxypregnanolone. The area on the thin layer chromatogram with the mobility of etiocholanolone was chromatographed on paper in the ligroin C : propylene glycol system. Scanning indicated a large area of radioactivity at the starting line as well as a smaller area with the mobility of etiocholanolone. The large area at the starting line was rechromatographed in the toluene : propylene glycol system. In this system the major fraction of the radioactivity had the mobility of 17α-hydroxypregnanolone. The area which had the mobility of 17α-hydroxypregnanolone on T.L.C. was chromatographed on paper in the toluene : Propylene glycol system. Scanning of the paper indicated a zone of radioactivity which corresponded to the standard 17α-hydroxypregnanolone run on the same paper. The zone was eluted. To the combined 17α-hydroxypregnanolone eluates were added 110.96 mg of non-radioactive carrier. The mixture was then crystallized twice from ether-methanol, and constant specific activity was achieved in the crystals and mother liquors. The crystals and mother liquor from

Figure VI

ELUTION PATTERNS OF RADIOACTIVITY FROM SILICA GEL COLUMNS OF EXPERIMENT IV.

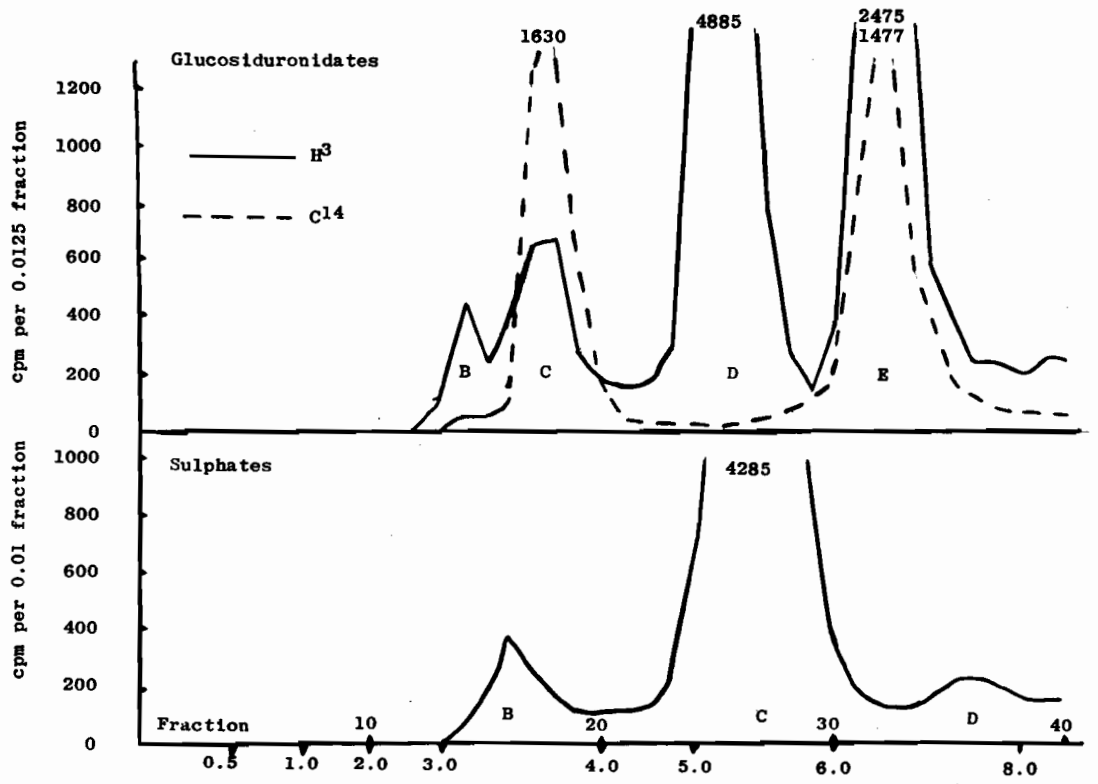
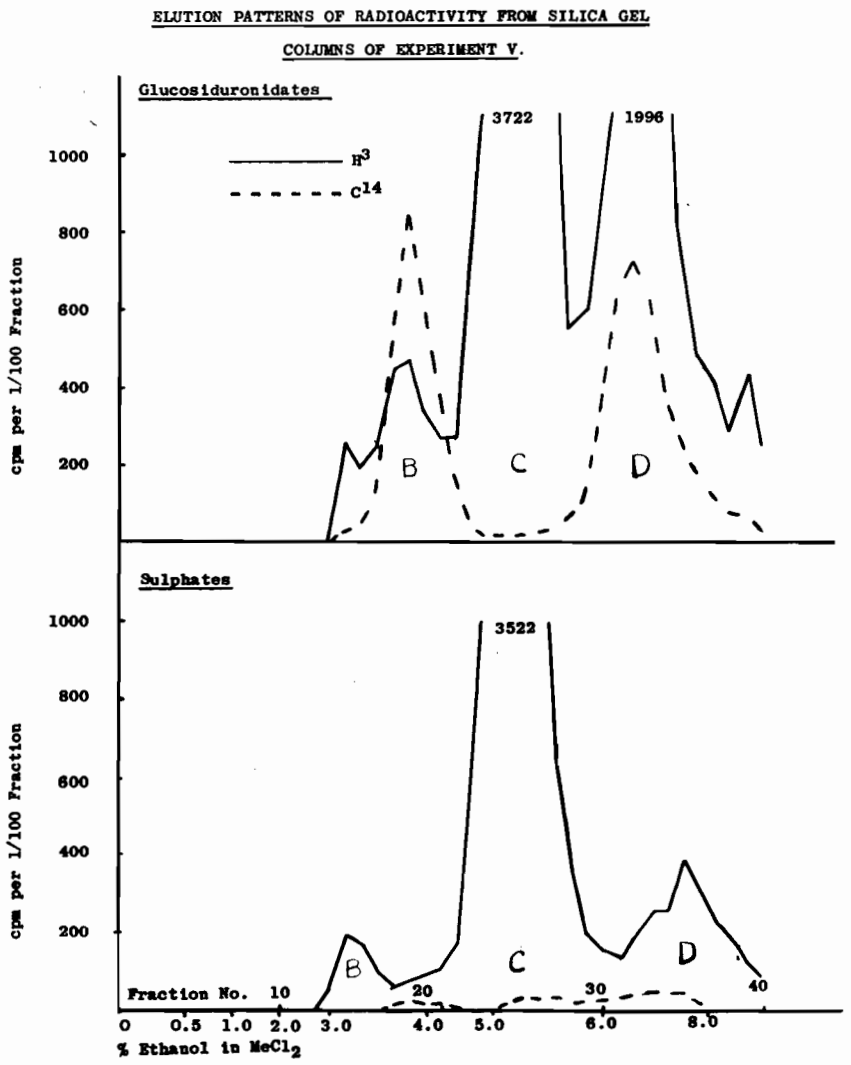


Figure VII


ELUTION PATTERNS OF RADIOACTIVITY FROM SILICA GEL
COLUMNS OF EXPERIMENT V.
Glucosiduronidates
H3
C14
3722
1996
B
C
D
cpm per 1/100 Fraction
1000
800
600
400
200
Sulphates
3522
B
C
D
Fraction No. 10
20
30
40
0
0.5
1.0
2.0
3.0
4.0
5.0
6.0
8.0
% Ethanol in MeCl2

the second crystallization were combined and reduced with sodium borohydride which was followed by oxidation with sodium bismuthate. The resulting etiocholanolone was then chromatographed on alumina and crystallized from ligroin-ether and isooctane-ether. The final crystals and mother liquor were combined and acetylated to form etiocholanolone acetate which was crystallized from ligroin-ether to constant specific activity.

Peak D from Experiment IV column of the glucosiduronidate extract was chromatographed on T.L.C. The areas corresponding to the pregnenetriol standards were eluted and run on the partition type silica gel columns followed by paper chromatography of the radioactive fractions in the system ligroin : toluene : methanol : water (50 : 50 : 70 : 30). Scanning of the papers showed peaks of radioactivity with the mobilities of the 20 and 20 isomers of pregnenetriol. The radioactive areas from the paper chromatograms with the mobility of Δ^5-pregnene-3β,17α,20α-triol were eluted and combined. Following the addition of 18.32 mg non-radioactive steroid to 470,800 cpm (H^3), the mixture was percolated through a silica gel column and crystallized twice. The specific activities of the crystals and mother liquor from the second crystallization did not differ significantly and were therefore combined and acetylated. The resulting Δ^5-pregnene-3β,17α,20α-triol-3,20-diacetate was percolated through a silica gel column and crystallized twice. The area on the paper chromatogram with the mobility of Δ^5-pregnene-3β,17α,20β-triol was eluted and non-radioactive steroid was added. Following percolation through a silica gel column the steroid was crystallized twice. Practically all the radioactivity

was in the two mother liquors so the compound was not purified further. That the radioactivity might not be Δ^5-pregnene-3β,17α,20β-triol prior to crystallization was indicated by the presence of considerable C^{14} which would not be expected in a compound with 3β-hydroxy-Δ^5 structure.

In Experiment IV, Peak E from the glucosiduronidate column was chromatographed on T.L.C. The area with the mobility of pregnanetriol was eluted and run on the Celite column containing boric acid in the stationary phase. One large radioactive peak was eluted and to the combined fractions in this peak were added 91.74 mg of carrier pregnanetriol. The mixture was crystallized twice from methanol-ether. The specific activities of the crystals and mother liquor were the same following the second crystallization so they were combined and oxidized with sodium bismuthate. The resulting etiocholanolone was crystallized twice and then acetylated. The acetate was crystallized from ligroin-ether and methanol-ether.

Peak C from the sulphate silica gel column of Experiment IV was chromatographed by T.L.C. The areas corresponding to the two pregnenetriol standards (20α and 20β isomers) were eluted and chromatographed on the silica gel partition type column. The peaks of radioactivity in the eluates of these columns were run on paper in the ligroin : toluene : MeOH:H_2O (50 : 50 : 70 : 30) system. The radioactive area with the mobility of Δ^5-pregnene-3β,17α,20α-triol was eluted and 6.42 mg non-radioactive steroid were added to 707,950 cpm (H^3). After percolation through a silica gel column, three crystalliza-

tions were required to attain a constant specific activity. The crystals and third mother liquor were combined and acetylated. The diacetate was percolated through a small column and crystallized twice. On none of the paper chromatograms of these column fractions was there any radioactivity with the mobility of Δ^5-pregnene-3β,17α,20β-triol. Peak B from the sulphate extract silica gel column contained 17-ketosteroids; these were not purified during this study. Peak D from the same column was chromatographed on paper in the ligroin : toluene : methanol : water system. Scanning indicated a peak of radioactivity with a mobility much less than that of Δ^5-pregnenetriol. This material was not examined further.

In Experiment V the fractions from the initial silica gel columns were combined according to a plot of the radioactivity content and treated in the same manner as the corresponding fractions in Experiment IV. Peak B from the glucosiduronidate column was chromatographed on thin layer plates and the area corresponding to etiocholanolone was eluted. This eluate was then chromatographed on paper in the ligroin C : propylene glycol system. A scan of the paper indicated three radioactive areas, one corresponding to the androsterone standard, one to the etiocholanolone standard and the largest one located at the starting line. This result indicated that the 17α-hydroxypregnanolone was spreading over a large area on the thin layer chromatogram. This also occurred in Experiment IV. The etiocholanolone and androsterone fractions were not purified further. The large peak of radioactivity on the starting line of the ligroin C : propylene glycol chromatogram was rechromatographed in the toluene : propylene glycol system

where the major fraction of the radioactivity had the mobility of 17α-hydroxypregnanolone. The area of the thin layer chromatograms corresponding to the 17α-hydroxypregnanolone standard was also eluted and run on paper in the toluene : propylene glycol system. The radioactivity which behaved as 17α-hydroxypregnanolone on this paper was combined with that eluted from the above paper chromatogram. A total of 78.38 mg of non-radioactive 17α-hydroxypregnanolone was added and following chromatography on an adsorption type silica gel column, it was crystallized twice. The specific activities of the crystals and second mother liquor did not differ significantly and they were combined, reduced with sodium borohydride and oxidized with sodium bismuthate to yield etiocholanolone. The etiocholanolone was crystallized twice after it had been percolated through an alumina column. The crystals and second mother liquor had the same specific activity and were combined and acetylated. The etiocholanolone acetate was percolated through an alumina column and crystallized twice.

The second peak of radioactivity from the initial silica gel column (Peak C) of the glucosiduronidate extract in Experiment V was chromatographed on thin layer plates. The areas corresponding to the pregnenetriol standards were chromatographed on the partition type silica gel column and the peaks of radioactivity eluted were run on paper in the ligroin : toluene : methanol : water (50 : 50 : 70 : 30) system. The areas corresponding to the two pregnenetriols were located by scanning for radioactivity and they were then eluted. The areas with the mobility of Δ^5-pregnene-3β,17α,20α-triol contained 783,165 cpm of

tritium. After the addition of 18.73 mg of non-radioactive steroid, the pregnenetriol was chromatographed on a silica gel column and crystallized twice. The crystals and second mother liquor were combined and acetylated. The pregnenetriol-3,20-diacetate was chromatographed on silica gel and crystallized twice. The areas of radioactivity on the paper chromatograms with the mobility of the Δ^5-pregnene-3β,17α,20β-triol standard were treated as described for those in the glucosiduronidate fraction of Experiment IV. Again, the major fraction of the radioactivity appeared in the mother liquors.

In Experiment V, peak D from the silica gel column of the glucosiduronidate extract was chromatographed on thin layer plates and the area with the mobility of pregnanetriol was eluted and chromatographed on a partition type silica gel column. A large peak of radioactivity was eluted and to it was added 92.57 mg of non-labelled pregnanetriol. Following percolation through a silica gel column it was crystallized twice and the crystals and a mother liquor had specific activities which did not differ significantly. The crystals and second mother liquor were combined and oxidized with sodium bismuthate to form etiocholanolone. After chromatography on alumina the etiocholanolone was crystallized twice. The crystals and second mother liquor had the same specific activity so they were combined and acetylated. The etiocholanolone acetate was percolated through an alumina column and crystallized twice.

Peak C from the silica gel chromatogram of the sulphate extracts of Experiment V was chromatographed on thin layer plates and the areas

corresponding to pregnenetriol were eluted and chromatographed first on a partition type silica gel column and then on paper in the ligroin : toluene : methanol : water system in order to separate the 20α and 20β isomers. The radioactive areas with the mobility of Δ^5-pregnene-3β,17α,20α-triol and Δ^5-pregnene-3β,17α,20β-triol were eluted. The eluted 20α isomer had 737,430 cpm of H^3 and to it was added 14.98 mg of carrier steroid. After percolation through a silica gel column, the pregnenetriol was crystallized twice. The crystals and second mother liquor were combined and acetylated. The diacetate was chromatographed on a silica gel column and crystallized twice. The area on the paper chromatogram with the mobility of the 20β-isomer of pregnenetriol was eluted and carrier steroid was added. After percolation through a silica gel column the mixture was crystallized twice. In this instance also, the larger fraction of the radioactivity appeared in the mother liquors. Peak B from the initial silica gel column contained the 17-ketosteroids which were not purified further. Peak D from the same column was not examined.

RESULTS

URINARY EXCRETION OF 17—KETOSTEROIDS

Prior to operation, a 24 hour urine specimen was collected and analysed for the individual 17-ketosteroids. The neutral extract of the urine obtained after treatment with β-glucuronidase and solvolysis was chromatographed on alumina by the gradient elution technique. The 17-ketosteroid excretion pattern is shown in Figure VIII.

Dehydroisoandrosterone was not detected and as compared to the normal female the most significant increases were in the 11-oxy-17-ketosteroid fractions. It was necessary to chromatograph the 11β-hydroxyandrosterone and 11-ketoetiocholanolone fraction on paper in the toluene : propylene glycol system to effect a good separation. The five 17-ketosteroids identified were androsterone, etiocholanolone, 11-ketoetiocholanolone, 11β-hydroxyandrosterone and 11β-hydroxyetiocholanolone. Two unidentified peaks of Zimmerman-positive material were eluted after the 11β-hydroxyetiocholanolone.

PURITY OF 17α-HYDROXYPREGNENOLONE-7α-H^3 and 17α-HYDROXYPROGESTERONE-4-C^{14}

As described in Part I, the 17α-hydroxypregnenolone-7α-H^3 was judged to be at least 97 per cent pure. The 17α-hydroxyprogesterone-4-C^{14} was at least 98 per cent pure as surmised from the specific activities of the crystals and unfractionated mother liquors resulting from the crystallization of 97,940 cpm of the labelled compound plus 103.7 mg of non-radioactive carrier. None of the specific activities of the crystals and mother liquors (see Table XV) differed significantly from the calculated value of 7,149 cpm/mg.

URINARY EXCRETION OF RADIOACTIVITY

In Experiment IV a total of 53.7 per cent of the administered tritium radioactivity and 62.9 per cent of the Carbon^{14} radioactivity were extracted from the urine following β-glucuronidase treatment and solvolysis (Table XVI). The corresponding figures for Experiment V were 57.7 per cent of the tritium and 78.0 per cent of the Carbon^{14} (Table XVII). In both experiments a larger fraction of the excreted

Figure VIII.

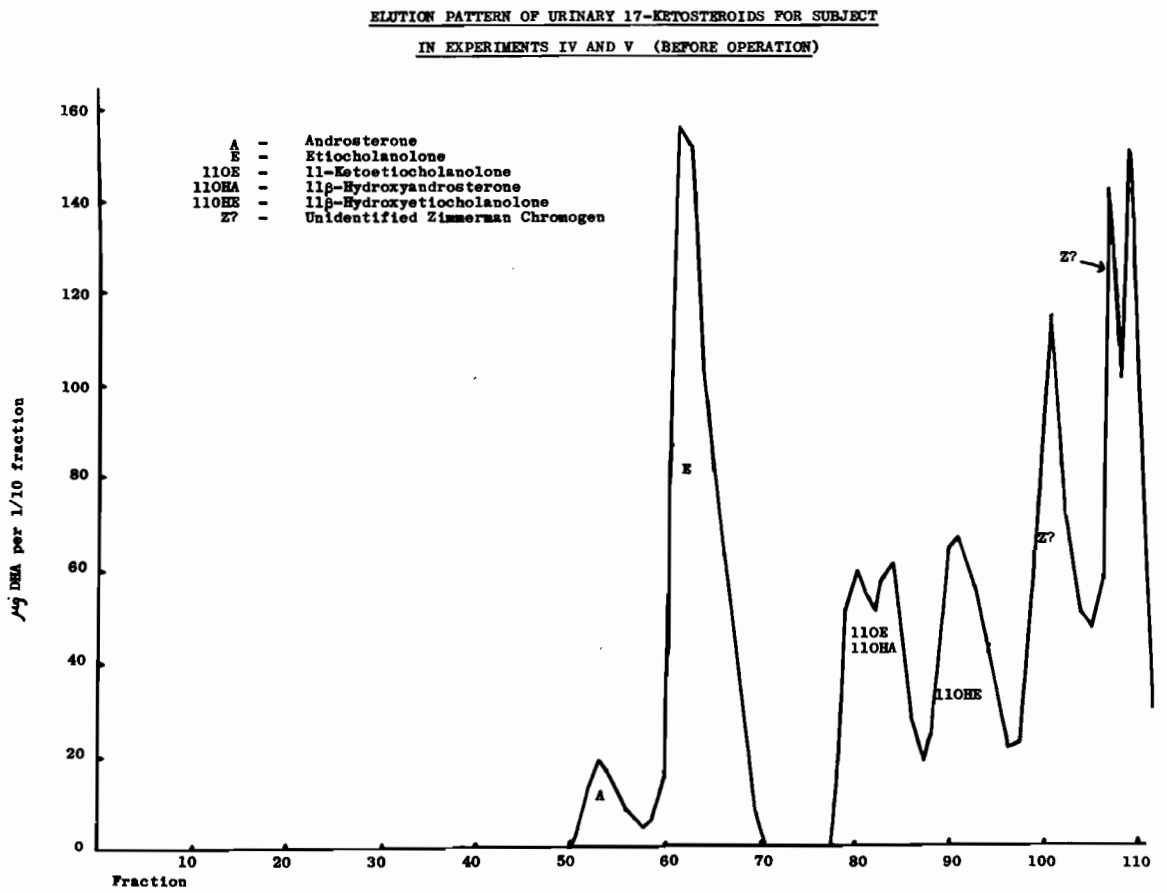

ELUTION PATTERN OF URINARY 17-KETOSTEROIDS FOR SUBJECT
IN EXPERIMENTS IV AND V (BEFORE OPERATION)
A - Androsterone
E - Etiocholanolone
11OE - 11-Ketoetiocholanolone
11OHA - 11β-Hydroxyandrosterone
11OHE - 11β-Hydroxyetiocholanolone
Z? - Unidentified Zimmerman Chromogen
μg DHA per 1/10 fraction
Fraction
0
20
40
60
80
100
120
140
160
10
20
30
40
50
60
70
80
90
100
110
A
E
11OE
11OHA
11OHE
Z?
Z?

TABLE XV.

TEST OF RADIOCHEMICAL HOMOGENEITY OF 17α-HYDROXYPROGESTERONE-4-C^{14}

Crystallization	Solvent	Specific Activities (cpm/mg) xlls	ml
1	Ligroin B-acetone	6869	6924
2	Methanol	6858	6460
3	Acetone	7118	6963
Calculated Specific Activity		7149	

TABLE XVI

RADIOACTIVITY EXTRACTED FROM URINE AFTER β-GLUCURONIDASE HYDROLYSIS AND SOLVOLYSIS IN EXPERIMENT IV

(administered 7.97 x 10^6 cpm of 17α-hydroxypregnenolone-7α-H^3 and 1.06 cpm 17α-hydroxyprogesterone-4-C^{14})

Glucosiduronidates

Day	H^3 x 10^3	% of dose	C^{14} x 10^3	% of Dose
1	1576	19.8	571	53.7
2	384	4.8	50	4.7
3	155	1.9	10	1.0
4	87	1.1	3	0.3
	2202	27.6	634	59.7

Sulphates

Day	H^3 x 10^3	% of Dose	C^{14} x 10^3	% of Dose
1	1565	19.6	30	2.8
2	387	4.9	5	0.4
3	106	1.3	-	
4	26	0.3	-	
	2084	26.1	35	3.2
TOTAL	4286	53.7	669	62.9

TABLE XVII

RADIOACTIVITY EXTRACTED FROM URINE AFTER β-GLUCURONIDASE HYDROLYSIS AND SOLVOLYSIS IN

EXPERIMENT V

(administered 7.71 x 10^6 cpm 17α-hydroxypregnenolone-7α-H^3 and 1.04 x 10^6 cpm 17α-hydroxyprogesterone-4-C^{14})

Glucusiduronidates

Day	H^3 x 10^3	% of Dose	C^{14} x 10^3	% of Dose
1	1689	21.9	583	56.2
2	499	6.5	131	12.6
3	369	4.8	25	2.4
4	132	1.7	7	0.7
	2689	34.9	746	71.9

Sulphates

Day	H^3 x 10^3	% of Dose	C^{14} x 10^3	% of Dose
1	849	11.0	45	4.4
2	454	5.9	9	0.9
3	321	4.2	6	0.6
4	128	1.7	2	0.2
	1752	22.8	62	6.1
TOTAL	4441	57.7	808	78.0

carbon-14 radioactivity was extracted following β-glucuronidase treatment (over 90 per cent of the total excreted in both experiments). In Experiment IV approximately equal quantities of the tritium (27.6 and 26.1 per cent of the dose) were extracted following β-glucuronidase treatment and after solvolysis. In Experiment V more of the tritium radioactivity was extracted following β-glucuronidase treatment (34.9 per cent of the dose) than following solvolysis (22.8 per cent). Qualitatively, the overall patterns of excretion for the two studies were similar. Insignificant quantities of radioactivity were excreted on day 4 of the experiments, indicating that the 4-day urine collections were adequate for obtaining all of the labelled metabolites formed.

PATTERNS OF ELUTION OF RADIOACTIVITY FROM SILICA GEL COLUMNS

The patterns of distribution of radioactivity in the silica gel chromatograms of the glucosiduronidate extracts for Experiments IV and V were similar. The same was true of the sulphate extracts for the two experiments (Figures VI and VII). The amount of radioactivity and percentage of the injected radioactivity for each of the groups of combined fractions for the silica gel columns for Experiments IV and V are given in Tables XVIII and XIX respectively. The elution patterns of radioactivity from the silica gel columns for the glucosiduronidate extracts in Experiments IV and V are similar in that there were three principal peaks of radioactivity eluted. The first of these peaks (C in Experiment IV, and B in Experiment V) contained a small quantity (ca 2 — 3 per cent) of the tritium originally administered and approximately 25 per cent of the carbon-14 originally administered. The second peak of radioactivity

TABLE XIX

RADIOACTIVITY IN FRACTIONS COMBINED FROM SILICA GEL COLUMNS FOR EXPERIMENT V

Fractions	Radioactivity			
	cpm x 10^3		% of injected dose	
	H^3	C^{14}	H^3	C^{14}
		β-glucuronidase extract		
1 - 12	--	--	--	--
13 - 21	242.6	255.0	3.15	24.52
22 - 28	1346.5	26.0	17.46	2.50
29 - 43	1132.2	321.0	14.68	30.87
Total	2721.3	602.0	35.29	57.89
		Solvolysis extract		
1 - 10	--	--	--	--
11 -- 17	41.3	1.6	0.54	0.02
18 - 31	1232.0	20.5	15.98	1.97
32 - 40	168.4	17.4	2.18	1.67
41 - 48	84.9	7.0	1.10	0.67
Total	1526.6	46.4	18.80	4.33

TABLE XVIII

RADIOACTIVITY IN FRACTIONS COMBINED FROM SILICA GEL COLUMNS FOR EXPERIMENT IV

Fractions	Radioactivity			
	H^3 (cpm x 10^3)	C^{14}	H^3 % of injected dose	C^{14}
		β-glucuronidase extract		
1 - 12	--	--	--	--
13 - 15	21.6	57.6	0.27	0.54
16 - 22	181.0	274.5	2.27	25.90
23 - 29	881.0	10.0	11.05	0.9
30 - 38	690.0	324.0	8.66	30.57
39 - 42	45.8	14.2	0.57	0.13
Total	1819.4	680.3	22.82	57.23
		Solvolysis extract		
1 - 12	1.8			
13 - 19	133.8	4.6	1.69	0.04
20 - 33	1476.8	14.4	21.10	0.14
34 - 44	156.6	14.8	1.48	0.14
45 - 58	207.0	9.4	1.95	0.09
59 - 61	124.0	0.5	1.56	-
Total	2100.0	43.7	27.87	0.41

(D in Experiment IV and C in Experiment V) contained a small quantity of carbon-14 and was the largest peak of the three with respect to tritium. The third major peak (E in Experiment IV, and D in Experiment V) contained both tritium and carbon-14 with the latter predominating. Only one significant peak of radioactivity was eluted from the silica gel columns of the sulphate extracts (peak C for both experiments) and this peak was predominantly tritium.

SPECIFIC ACTIVITIES OF THE ISOLATED COMPOUNDS AND THEIR DERIVATIVES

Following the various chromatographic steps for the purification of the individual steroid, non-labelled carrier was added and they were percolated through silica gel columns and crystallized several times until the specific activities of the crystals and unfractionated mother liquors did not differ significantly. The crystals and final mother liquor for each compound were then combined and a derivative was prepared, purified on a column and crystallized. The pregnanetriol was reacted with sodium bismuthate to form etiocholanolone. After crystallization of the etiocholanolone, etiocholanolone acetate was prepared. The isolated 17α-hydroxypregnanolone sample was treated in a similar manner. Before sodium bismuthate treatment, the 17α-hydroxypregnanolone was reduced with sodium borohydride and reacted with $NaBIO_3$ without purification. Sodium bismuthate oxidation of Δ^5-pregnenetriol does not yield dehydroisoandrosterone and as a result, the diacetate served as a derivative in the purification procedure.

Non-radioactive carrier steroid was added to each of the isolated metabolites prior to crystallization. In Experiment IV, 91.74 mg of

pregnanetriol was added to 546,800 cpm H^3 and 271,200 cpm C^{14}, 92.36 mg of 17α-hydroxypregnanolone was added to 91,400 cpm H^3 and 224,400 cpm C^{14}, 18.32 mg of Δ^5-pregnene-3β,17α,20α-triol was added to 470,800 cpm H^3 (glucosiduronidate fraction) and 6.42 mg to 707,950 cpm H^3 (sulphate fraction). The specific activities of the crystals and mother liquors of the free compounds and their derivatives are shown in Tables XX to XXII. In all cases, the specific activities of the derivatives were the same as the calculated values within the limits of experimental error.

In Experiment V, 92.57 mg of carrier pregnanetriol was added to 815,600 cpm H^3 and 320,800 cpm C^{14}, 78.38 mg of 17α-hydroxypregnanolone was added to 81,700 cpm H^3 and 170,700 cpm C^{14}, 18.73 mg of Δ^5-pregnene-triol was added to 783,165 cpm H^3 (glucosiduronidate fraction) and 14.98 mg was added to 737,430 cpm H^3 (sulphate fraction). The specific activities and H^3/C^{14} ratios for the free compounds and their derivatives are shown in Tables XXIII to XXV. In this experiment (V) the specific activities of the derivatives agreed with the calculated ones with one exception. The specific activity with respect to tritium of the etiocholanolone prepared from the 17α-hydroxypregnanolone was approximately 50 per cent of the expected value. However, the H^3/C^{14} ratio was not changed on formation of the acetate.

In Table XXVI are shown the crystallization data for the Δ^5-pregnene-3β,17α,20β-triol samples for the two experiments. I_n no instance was a constant specific activity even approached.

The final specific activities of the three metabolites (based on those of the last derivative formed) the weight of carrier added to each sample and the minimal per cent conversions of the labelled precursors to the three urinary products are shown in Table XXVII. In both experi-

TABLE XX

PROOF OF RADIOCHEMICAL HOMOGENEITY OF 17α-HYDROXYPREGNANOLONE ISOLATED FROM GLUCUSIDURONIDATE FRACTION FROM EXPERIMENT IV

	Specific Activities -- cpm/mg. First Crystallization			Second Crystallization		
Free	820 cpm H^3	) 0.38*		693 cpm H^3	) 0.59	
	2162 cpm C^{14}	)		1174 cpm C^{14}	)	
	817 cpm H^3	) 0.38		808 cpm H^3	) 0.42	
	2130 cpm C^{14}	)		1941 cpm C^{14}	)	
Reduction and Oxidation Product (Etiocholanolone)	861 cpm H^3	) 0.36		800 cpm H^3	) 0.37	
	2362 cpm C^{14}	)		2153 cpm C^{14}	)	
	828 cpm H^3	) 0.35		799 cpm H^3	) 0.33	
	2384 cpm C^{14}	)		2451 cpm C^{14}	)	
	Calculated: 944 cpm H^3	) 0.38				
	2460 cpm C^{14}	)				
Eitocholanolone Acetate	690 cpm H^3	) 0.31		694 cpm H^3	) 0.30	
	2197 cpm C^{14}	)		2145 cpm C^{14}	)	
	676 cpm H^3	) 0.31		661 cpm H^3	) 0.30	
	2213 cpm C^{14}	)		2226 cpm C^{14}	)	
	Calculated: 727 cpm H^3	) 0.35				
	2070 cpm C^{14}	)				

TABLE XXI

PROOF OF RADIOCHEMICAL HOMOGENEITY OF PREGNANETRIOL ISOLATED FROM GLUCUSIDURONIDATE FRACTION FROM EXPERIMENT IV

	Specific Activities (cpm/mg)			
	First Crystallization		Second Crystallization	
	Crystals	ML	Crystals	ML
Free	5254 cpm H^3) 2.08* 2521 cpm C^{14})	4450 cpm H^3) 2.17 2053 cpm C^{14})	5436 cpm H^3) 1.99 2727 cpm C^{14})	5664 cpm H^3) 2.03 2787 cpm C^{14})
Oxidation product (etiocholanolone)	6133 cpm H3) 1.92 3203 cpm C^{14})	5764 cpm H^3) 1.93 2987 cpm C^{14})	6158 cpm H^3) 1.94 3173 cpm C^{14})	6009 cpm H^3) 1.92 3135 cpm C^{14})
	Theoretical: 6300 cpm H^3) 3160 cpm C^{14}) 1.99			
Etiocholanolone acetate	5036 cpm H^3) 1.88 2673 cpm C^{14})	4788 cpm H^3) 1.88 2549 cpm C^{14})	5090 cpm H^3) 1.85 2751 cpm C^{14})	5153 cpm H^3) 1.91 2705 cpm C^{14})
	Theoretical: 5373 cpm H^3) 2770 cpm C^{14}) 1.94			

* ratio of H3 and C^{14}.

TABLE XXII

PROOF OF RADIOCHEMICAL HOMOGENEITY OF Δ^5-PREGNENE-3β,17α,20α-TRIOL ISOLATED FROM GLUCOSIDURONIDATE AND SULPHATE FRACTIONS OF EXPERIMENT IV

Crystallization	Specific Activities - (cpm H^3/mg)			
	Free Compound		Diacetate	
	Crystals	ML	Crystals	ML
			Glucosiduronidate Fraction	
1	23,652	16,831	19,935	18,768
2	23,063	22,299	19,658	19,872
Theoretical			17,989	
			Sulphate Fraction	
1	85,046	29,643	74,957	65,631
2	92,760	60,809	75,770	75,543
3	93,888	90,688		
Theoretical			73,233	

TABLE XXIII

PROOF OF RADIOCHEMICAL HOMOGENEITY OF PREGNANETRIOL ISOLATED FROM GLUCOSIDURONIDATE FRACTION FROM EXPERIMENT V

	Specific Activities (cpm/mg)			
	First Crystallization		Second Crystallization	
	Crystals	ML	Crystals	ML
Free	7449 cpm H^3) 2.30 3244 cpm C^{14})	5671 cpm H^3) 2.67 2122 cpm C^{14})	7222 cpm H^3) 2.47 2928 cpm C^{14})	7366 cpm H^3) 2.59 2847 cpm C^{14})
Oxidation Product (etiocholanolone)	8497 cpm H^3) 2.49 3408 cpm C^{14})	7872 cpm H^3) 2.44 3223 cpm C^{14})	8620 cpm H^3) 2.48 3480 cpm C^{14})	8274 cpm H^3) 2.51 3293 cpm C^{14})
		Theoretical: 8622 cpm H^3) 2.47 3455 cpm C^{14})		
Etiocholanolone acetate	7440 cpm H^3) 2.41 3089 cpm C^{14})	6911 cpm H^3) 2.42 2891 cpm C^{14})	7392 cpm H^3) 2.46 3004 cpm H^{14})	7134 cpm H^3) 2.43 2937 cpm C^{14})
		Theoretical: 7338 cpm H^3) 2.44 3010 cpm C^{14})		

TABLE XXIV

PROOF OF RADIOCHEMICAL HOMOGENEITY OF 17α-HYDROXYPREGNANOLONE ISOLATED FROM GLUCODISURONIDATE FRACTION FROM EXPERIMENT V

	Specific Activities (cpm/mg)			
	First Crystallization		Second Crystallizations	
	Crystals	ML	Crystals	ML
Free	1306 cpm H^3) 0.62* 2091 cpm C^{14})	1470 cpm H^3) 0.93 1576 cpm C^{14})	1241 cpm H^3) 0.62 2000 cpm C^{14})	1360 cpm H^3) 0.74 1825 cpm C^{14})
Reduction and Oxidation Product (Etiocholanolone)	679 cpm H^3) 2) 0.29 2312 cpm C^{14})	569 cpm H^3) 0.32 1750 cpm C^{14})	667 cpm H^3) 0.29 2312 cpm C^{14})	735 cpm H^3) 0.33 2221 cpm C^{14})
	Theoretical: 1433 cpm H^3) 0.62 2310 cpm C^{14})			
Etiocholanolone	616 cpm H^3) 0.32 1952 cpm C^{14})	580 cpm H^3) 0.30 1917 cpm C^{14})	590 cpm H^3) 0.30 1978 cpm C^{14})	574 cpm H^3) 0.30 1941 cpm C^{14})
	Theoretical: 582 cpm H^3) 0.29 2004 cpm C^{14})			

* Ratio of H^3 to C^{14}

TABLE XXV

PROOF OF RADIOCHEMICAL HOMOGENEITY OF Δ^5-PREGNENE-3β,17α,20α-TRIOL ISOLATED FROM GLUCOSIDURONIDATE AND SULPHATE FRACTIONS OF EXPERIMENT V

Crystallization	Specific Activities - (cpm H^3/mg)			
	Free Compound		Diacetate	
	Crystals	ML	Crystals	ML
		Glucosiduronidate Fraction		
1	34,658	20,378	29,187	29,969
2	37,742	36,677	30,178	29,579
Theoretical			29,439	
		Sulphate Fraction		
1	28,310	17,664	24,379	22,444
2	28,612	27.029	24,426	23,567
Theoretical			22,317	

TABLE XXVI

CRYSTALLIZATION DATA FOR Δ^5-PREGNENE-3β,17α,20α-TRIOL

FRACTIONS

Crystallization	Specific Activities (cpm/mg) Crystals	ML
	Experiment IV - Glucosiduronidate Fraction	
1	98 cpm H^3 14 cpm C^{14}	366 cpm H^3 526 cpm C^{14}
2	13 cpm H^3 3 cpm C^{14}	78 cpm H^3 87 cpm C^{14}
	Experiment V - Glucosiduronidate Fraction	
1	301 cpm H^3 26 cpm C^{14}	2257 cpm H^3 492 cpm C^{14}
2	46 cpm H^3 --	933 cpm H^3 208 cpm C^{14}
	Experiment V - Sulphate Fraction	
1	171 cpm H^3 34 cpm C^{14}	5246 cpm H^3 1179 cpm C^{14}
2	84 cpm H^3 --	1727 cpm H^3 370 cpm C^{14}

TABLE XXVII

FINAL SPECIFIC ACTIVITIES AND WEIGHTS OF CARRIER ADDED TO METABOLITES ISOLATED AND MINIMAL PER CENT CONVERSIONS OF 17α-HYDROXYPREGNENOLONE-7α-H^3 AND 17α-HYDROXYPROGESTERONE-4-C^{14} TO URINARY PREGNANETRIOL, 17α-HYDROXYPREGNANOLONE AND Δ^5-PREGNENE-3β,17α,20α-TRIOL

	Experiment IV			Experiment V		
	Specific Activity (cpm/mg)	Weight of Carrier (mg.)	Minimal % Conversion	Specific Activity (cpm/mg)	Weight of Carrier (mg.)	Minimal % Conversion
Pregnanetriol	5309 cpm H^3 2737 cpm C^{14}	91.74	6.11 % H^3 23.60 % C^{14}	7303 cpm H^3 2968 cpm C^{14}	92.57	8.77 % H^3 26.47 % C^{14}
17α-Hydroxy pregnanolone	672 cpm H^3 2200 cpm C^{14}	92.36	0.78 % H^3 19.10 % C^{14}	657 cpm H^3 1968 cpm C^{14}	78.38	0.67 % H^3 14.86 % C^{14}
Δ^5-Pregnene-3β,17α, 20α-triol						
A. Glucosiduronidate	25,203 cpm H^3	18.32	5.8 % H^3	38,690 cpm H^3	18.73	9.40 % H^3
B. Sulphate Fraction	97,141 cpm H^3	6.42	7.83 % H^3	31,315 cpm H^3	14.98	6.09 % H^3

ments approximately 40 per cent of the administered C^{14} was isolated as 17 α-hydroxypregnanolone and pregnanetriol. Only about 7 per cent of the tritium was isolated in the form of these two metabolites. Approximately 15 per cent of the administered tritium was isolated as Δ^5-pregnene-3β,17α,20α-triol in the two experiments.

DISCUSSION

The minimal per cent conversions of 17 α-hydroxypregnenolone-7 α-H^3 and 17α-hydroxyprogesterone-4-C^{14} to urinary pregnanetriol; 17α-hydroxypregnanolone and Δ^5-pregnene-3β,17α,20α-triol were measured in a 19-year-old female subject with Cushing's syndrome due to bilateral adrenal hyperplasia. The experiment was performed both before adrenalectomy and again after adrenalectomy. These results are minimal in that no corrections were made for losses occurring during the isolation procedures. To establish the radiochemical homogeneity of the isolated urinary metabolites, carrier steroid was added and crystallizations were performed on the free compound as well as on derivatives of the free steroid. The procedures are capable of removing radioactive contaminants as was evidenced by the loss of approximately 50 per cent of the tritium in the 17α-hydroxypregnanolone of Experiment V (Figure XXV) during preparation of the derivative, etiocholanolone. This labelled impurity had not been removed by the crystallization of the 17α-hydroxypregnanolone itself or during the chromatography prior to crystallization.

One of the primary objectives of this study was to compare the relative contributions of 17α-hydroxypregnenolone and 17α-hydroxypro-

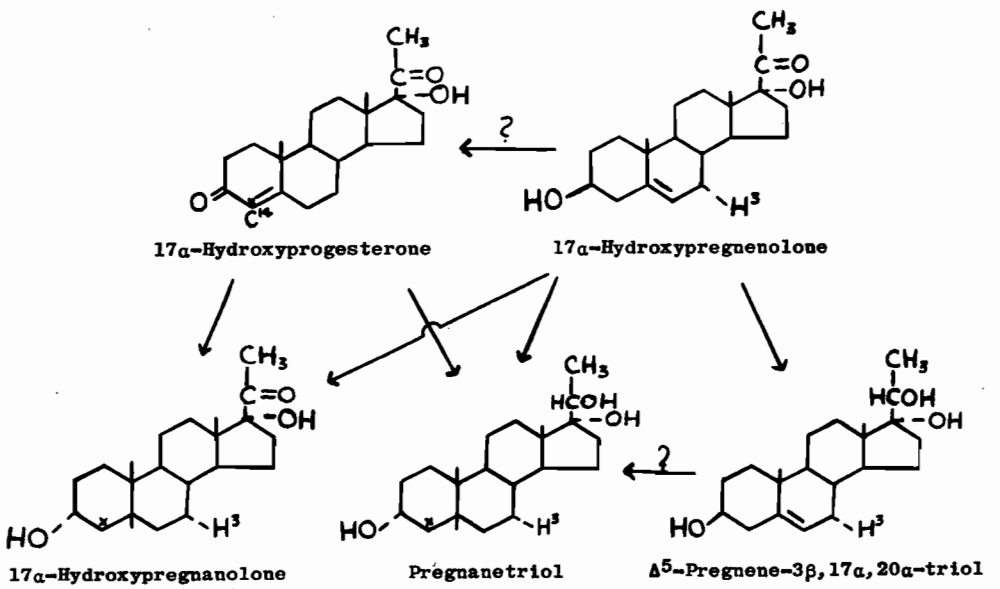
gesterone to urinary pregnanetriol and 17α-hydroxypregnanolone. The H^3/C^{14} ratio of the administered steroid mixture was approximately 7.5 to 1. In Experiment IV the H^3/C^{14} ratio in the urine pregnanetriol was 1.8 to 1 indicating that four times more 17α-hydroxyprogesterone than 17α-hydroxypregnenolone was converted to pregnanetriol. This difference in the rate of metabolic transformation is also reflected in the measured conversions (6.11 per cent of the administered 17α-hydroxy-pregnenolone-7α-H^3 and 23.60 per cent of the 17α-hydroxyprogesterone-4-C^{14} were excreted as pregnanetriol). In Experiment V, the H^3/C^{14} ratio in the urinary pregnanetriol was approximately 2.5 to 1. The conversion of 17α-hydroxypregnenolone to pregnanetriol was slightly more efficient than in Experiment IV. The conversion of 17α-hydroxypregnenolone to urine 17α-hydroxypregnanolone occurred only to a minor extent. The H^3/C^{14} ratio in this urine steroid was approximately 0.3 in both experiments. Of the isolated urine metabolites of the 17α-hydroxy-pregnenolone-7α-H^3, Δ^5-pregnene-3β,17α,20α-triol was the most important. Approximately 15 per cent of the administered dose was isolated from the urine in the form of this metabolite. This metabolite was found in the extracts obtained after β-glucuronidase treatment and in those obtained after solvolysis. In Experiment IV, more of it was isolated from the sulphate extract than from the glucosiduronidate extract. The reverse was found in Experiment V. No explanation can be offered for this difference.

The metabolism of the administered 17α-hydroxypregnenolone-7α-H^3 and the 17α-hydroxyprogesterone-4-C^{14} is summarized in Figure IX. As stated above, the H^3/C^{14} ratio in the urine pregnanetriol was only

Figure IX.

PERIPHERAL METABOLISM OF 17α-HYDROXYPREGNENOLONE-7α-H^3 AND 17α-HYDROXYPROGESTERONE-4-C^{14}

	Injected H^3/C^{14}
Pre-op	7.52
Post-op	7.41



Isolated	H^3/C^{14}	H^3/C^{14}
Pre-op	0.35	1.94
Post-op	0.29	2.44

one quarter of that of the administered mixture. This indicated that of the two precursors 17∝-hydroxyprogesterone was more efficiently converted to pregnanetriol. The H^3/C^{14} ratios of the urinary 17∝-hydroxypregnanolone were 1/6 to 1/8 of the H^3/C^{14} ratios in the urine pregnanetriol. This indicates that the two urine steroids do not share a single common precursor. The greater part of the 17∝-hydroxypregnenolone going to pregnanetriol was not converted via 17∝-hydroxyprogesterone, if the latter is the direct precursor of 17∝-hydroxypregnanolone. The most reasonable explanation for the higher H^3/C^{14} ratio in the pregnanetriol is the conversion of 17∝-hydroxypregnenolone to pregnanetriol via Δ^5-pregnenetriol. If this conversion were as efficient as the transformation of 17∝-hydroxyprogesterone to pregnanetriol, then the H^3/C^{14} ratio in the pregnanetriol should approach 7.5 to 1.

Pregnanetriol had long been considered as a unique metabolite of 17∝-hydroxyprogesterone. The conversion of 17∝-hydroxypregnenolone to pregnanetriol was first reported by Fotherby (248). Roberts et al (120) studied the conversions of labelled 17∝-hydroxypregnenolone to urinary pregnanetriol and Δ^5-pregnene-3β,17∝,20∝-triol in a subject with an adrenal adenoma. They measured the specific activities of the two urinary metabolites and found them to be the same within the limits of experimental error. They concluded that in their subject 17∝-hydroxypregnenolone was the major precursor of pregnanetriol as a second significant source would have lowered its specific activity. Fukushima et al (117) studied the metabolism of labelled 17∝-hydroxyprogesterone in a subject with adrenal carcinoma. The specific activity of the

urinary 17α-hydroxypregnanolone was ten times greater than that of the pregnanetriol. This result indicated that some other steroid was the major precursor of urinary pregnanetriol. These authors suggested from the existing knowledge that the other precursor of the urinary pregnanetriol might be 17α-hydroxypregnenolone. In another study on a subject with adrenal carcinoma, Fukushima et al (205) administered tritium-labelled 17α-hydroxypregnenolone and C^{14}-labelled 17α-hydroxyprogesterone and measured the specific activities of urinary pregnanetriol, 17α-hydroxypregnanolone and Δ^5-pregnene-3β,17α,20α-triol. The presence of C^{14} in the urinary pregnanetriol indicated that 17α-hydroxypregnenolone was not its sole precursor and the H^3/C^{14} ratio suggests that 17α-hydroxyprogesterone could serve as a significant precursor. The H^3/C^{14} ratio in the pregnanetriol was 2.17 as compared to the precursor ratio of 2.89. Both administered steroids were converted to pregnanetriol with almost equal efficiency. This study by Fukushima et al is the only one previously reported in which 17α-hydroxypregnenolone and 17α-hydroxyprogesterone were administered simultaneously. Fukushima et al (116, 205) have reported studies on the metabolism of the two individually in normal subjects. The studies of Roberts et al (120) and Fukushima et al (117) which have just been discussed utilized as experimental subjects, patients with an abnormally high excretion of pregnanetriol and Δ^5-pregnene-3β,17α,20α-triol. In these situations it is possible that the secretion of one of the normal precursors of pregnanetriol is elevated while the other or others are not. The conclusions drawn from studies in such patients

may not be applicable to normal subjects. The urinary excretion of pregnanetriol by the subject used in this study was within the normal range (1 mg.), although the excretions of other steroids were elevated. The results indicate that in this subject, 17α-hydroxyprogesterone was more efficient than 17α-hydroxypregnenolone as a precursor of urinary pregnanetriol.

The conversions of 17α-hydroxyprogesterone to pregnanetriol in Experiments IV and V (23.60 and 26.47 per cent, respectively) are comparable to those found in a normal subject (22.3 per cent) and in one with congenital adrenal hyperplasia (16.2 per cent) by Fukushima et al (116). The conversions were lower for two subjects with adrenal carcinoma (10.6 and 8.8 per cent). In one of the two subjects with adrenal carcinoma the conversion of 17α-hydroxypregnenolone to urinary pregnanetriol was 6.6 per cent. This compares favourable to those found in this report (6.11 and 8.77 per cent) and to those reported for the subject with an adrenal adenoma (6.4 per cent) reported by Roberts et al (120) and for a normal subject (5.1 per cent) reported by Fukushima et al. The conversions of 17α-hydroxyprogesterone to urinary 17α-hydroxypregnanolone by the subjects of this report (14.86 and 19.10 per cent) and by the normal subject (23.3 per cent) and the other subjects (13.3, 15.6 and 7.9 per cent) reported by Fukushima et al (116, 117, 205) are similar with the exception of the last subject of Fukushima et al. This subject had adrenal carcinoma. The conversions of 17α-hydroxypregnenolone to 17α-hydroxypregnanolone by our subject and those of Fukushima et al are all similar. The conversions of 17α-hydroxypregnenolone to Δ5-pregnenetriol ranged from 8.8 per cent

in the normal subject reported by Fukushima et al (205) to 22 per cent in the subject with the adrenal adenoma. The results for the subject in this report were 13.63 and 15.49 per cent in Experiments IV and V respectively.

The only differences between Experiments IV and V was in the slightly more efficient conversion of 17α-hydroxypregnenolone to pregnanetriol in Experiment V. Thus, it can be concluded that the presence of the hyperplastic adrenals and their high steroid excretion had no great significant effect on the conversions studied.

PART III

STUDIES ON THE URINARY EXCRETION OF 17-KETOSTEROIDS BY HOGS.

INTRODUCTION

Although there have been many studies on the urinary excretion of 17-ketosteroids by humans, there have been relatively few studies of this nature on animals. This fact has been noted by Dorfman and Shipley (260), by Wright (261) and by Short (262).

The data reported in the literature to date indicate that the excretion of 17-ketosteroids by most animals is very low. In older studies where total 17-ketosteroids, and not the individual compounds were measured colorimetrically on crude urine extracts, the results reported may be an over-estimation of the true values since it has been demonstrated by several workers that a large fraction of "Zimmerman chromogens" in some animal urine extracts is not steroidal in nature.

Danford and Danford (263) and Kimeldorf (264) measured total 17-ketosteroids in rabbit urine and found levels in the range of 1 - 2 mg per 24 hours. It was noted in later studies that depressions of the urine 17-ketosteroid levels following treatment with drugs was related to histologic changes in the adrenals, thus indicating that the adrenals are the source of at least part of the 17-ketosteroids. Glenn and Heftman (265) reported that the urinary excretion of neutral 17-ketosteroids of dogs was less than 1 mg per day. They found no sex difference in the levels of urinary 17-ketosteroids in dogs and the urinary titre was not increased by the administration of ACTH. No attempts were made in these studies to fractionate the 17-ketosteroids extract into its individual components.

Marker (266, 267) succeeded in isolating very small quantities of androsterone and dehydroisoandrosterone from the urine of bulls, cows and steers. The total concentration of 17-ketosteroids in the urine of cattle was found by Holtz (268) to be 0.3 mg per liter (maximum). Holtz noted that there was considerable non-steroidal Zimmerman-positive material in cattle urine. He was able to identify dehydroisoandrosterone, etiocholanolone and 11-ketoetiocholanolone but did not find a significant difference between bulls, steers, normal cows and cows with sexual abnormalities. Administered testosterone and ACTH resulted in an increased urinary 17-ketosteroid titre. Testosterone administrations produced a large increase in the excretion of urinary etiocholanolone while with ACTH the largest increase obtained was in the 11-ketoetiocholanolone fraction, with lesser increases in the dehydroisoandrosterone and etiocholanolone fractions. Androsterone was also found in the urine following ACTH administration.

The 17-ketosteroid level in pregnant goat's urine was found to be less than 1 mg per liter (269). Isoandrosterone was identified and there was chromatographic evidence for the presence of androsterone, etiocholanolone and 11-ketoetiocholanolone. The infra red spectra of the latter two were reported to be not well defined. Very small quantities of dehydroisoandrosterone and isoandrosterone have been detected in pregnant mare's urine (270).

The excretion of 17-ketosteroids by guinea pigs has been the subject of several studies. Peron and Dorfman (271) were able to identify 11β-hydroxyetiocholanolone, 11β-hydroxyandrosterone, 11-keto-

androsterone and 11-ketoetiocholanolone in urine collected from guinea pigs which were being stimulated with ACTH. Later, these same authors (272) reported that the quantities of 11β-hydroxyetiocholanolone and 11-ketoetiocholanolone excreted by guinea pigs were increased following ACTH stimulation, while the excretion of 6β,11β-dihydroxy-Δ^4-androstenedione was not. These results suggested that the adrenals were the source of the urinary 11-oxy-17-ketosteroids but that 6-hydroxylation occurred elsewhere. Brooks et al (273) reported that the urinary excretion of 17-ketosteroids in guinea pigs increased during pregnancy. Ovariectomies performed after 7 - 7½ weeks of pregnancy did not affect the increase in 17-ketosteroid excretion. The authors provided good evidence for the presence of isoandrosterone as well as the 11-oxy-17-ketosteroids in the urine extract.

Shubert and Wehrberger (274) isolated twelve steroids from the urine of female rats and identified them by infra red analysis. These included androsterone, etiocholanolone, and their 11-keto and 11β-hydroxy analogues, Δ^4-androstenedione, etiocholanedione, 11-keto-isoandrosterone and 11β-hydroxyisoandrosterone.

There are several reports in the literature on the androgens secreted in spermatic vein blood of animals. Suzuki and Eto (275) found that testosterone was the major androgen present in the spermatic vein blood of adult rats. The administration of HCG caused an eight-fold increase in the level of testosterone in the spermatic vein blood and a two-fold increase of the same hormone in the testes itself. Another C_{19} steroid, Δ^4-androstenedione, was also detectable in the

spermatic vein blood following HCG injections.

Lindner (276, 277, 278) and Lindner and Rowson (279) published a series of studies on the androgens secreted by farm animals. By analysing arterial and spermatic vein blood, testosterone, Δ^4-androstenedione and 17α-hydroxyprogesterone were identified as secretory products of bull testes. The ratio of testosterone to Δ^4-androstenedione was found to increase as the animal matured. The secretion of testosterone as well as the amount of hormone present in the testes were both increased by HCG administration. When calves were subjected to unilateral orchidectomy at the age of 1½ months, the rate of testosterone secretion at the age of 5 months by the remaining testis, was significantly greater than the combined output of the two testes in control animals of the same age. Testosterone and Δ^4-androstenedione were found in spermatic vein blood of rams, boars and stallions. HCG administration led to an increase of the testosterone levels in the spermatic vein blood of most of the experimental animals examined.

Although it has been reported that boar testes secrete considerable quantities of testosterone, earlier reports indicated a very low level of urinary excretion of 17-ketosteroids in this species. Green and Winters (280) determined the amounts of 17-ketosteroids in boar urine and obtained results ranging from 0.6 to 3 mg per 24 hours. There was a rise in the 17-ketosteroid levels with maturity. The urinary excretion also appeared to vary with the sexual activity of the animals.

In a search for an animal with a 17-ketosteroid excretion pat-

tern similar to that found in humans, in order that the liver of such a species could be used for in vitro experiments, we became interested in the recent reports of Huis in't Veld et al (281, 282), which indicated that the pig might be the most suitable. These investigators isolated dehydroisoandrosterone and 3-chloroandrost-5-ene-17-one (considered to be an artifact resulting from the acid hydrolysis of dehydroisoandrosterone sulphate) from male hog urine. Isoandrosterone was found in considerable quantities while androsterone and etiocholanolone were not present in significant amounts. Paper chromatographic evidence was also presented to indicate the presence of 11-oxy-17-ketosteroids.

A number of steroids have been isolated from hog adrenals and testes. A partial list of the steroids which have been isolated from these two endocrine tissues is presented in Table XXVIII. These include the classical adrenal secretion products (283, 284) and some of the biosynthetic intermediates (285). Several C_{19} steroids have been isolated from hog adrenals (286 - 288). Pregnenolone (289, 290) and two Δ^{16} steroids (291) have been isolated from hog testes. Prelog et al (292) concluded that testosterone was probably present in hog testes tissue.

The objects of the experiments to be described was to isolate and identify the 17-ketosteroids in urine collected from normal and gonadectomized male and female hogs and pregnant females and to measure the effects of ACTH and HCG on the urinary levels of the individual steroids in the normal male hog.

TABLE XXVIII

STEROIDS ISOLATED FROM HOG ADRENALS AND HOG TESTES

HOG ADRENALS

C_{21} Steroids

Hydrocortisone

Cortisone

11-Desoxyhydrocortisone

Corticosterone

Aldosterone

17α-Hydroxyprogesterone

17α-Hydroxypregnenolone

Progesterone

Pregnenolone

C_{19} Steroids

Δ^4-Androstenedione

11β-Hydroxyandrosterone

11β-Hydroxyisoandrosterone

11-Ketoisoandrosterone

HOG TESTES

Δ^5-pregnenolone

Δ^{16}-androsten -3α-ol

Δ^{16}-androsten -3β-ol

EXPERIMENTAL

METHODS

All the methods used in this study have been previously described in Parts I and II. The urines were first treated with β-glucuronidase to cleave the steroid glucosiduronidates and then by solvolysis to cleave the sulphates. The neutral extracts so obtained were then chromatographed on alumina using the gradient elution technique.

Because the extracts were always very heavy, most of them were divided into several parts for this stage of the purification . The peaks of Zimmerman-positive material eluted from the column were combined and chromatographed on paper. The 11-desoxy-17-ketosteroids were run in the ligroin C : propylene glycol system while the 11-oxy-17-ketosteroids were run in the toluene : propylene glycol system. Appropriate standards were run on one side of the chromatograms. The standards as well as a narrow strip from the center of the extract lane were spotted by the Zimmerman reaction. The Zimmerman-positive areas were eluted and partitioned between ethyl acetate and water to remove the last traces of propylene glycol. Since dehydroisoandrosterone and isoandrosterone do not separate in the ligroin C propylene glycol system, the area containing the two was rerun in the ligroin B:methanol : water (10 : 9 : 1) system which effects a clear separation. The fractions were then percolated through small alumina columns and portions submitted for infrared analysis. In certain

instances thin layer chromatography on silica gel G, using the solvent systems ethyl acetate : n-hexane (9 : 1) and ethyl acetate : isooctane (7 : 3) was used for additional purification. The 11-oxy-17-ketosteroids which were to be examined by infrared analysis in CS_2 were acetylated prior to percolation through the alumina columns. The free compounds have low solubility in CS_2 and they had to be examined in KBr pellets.

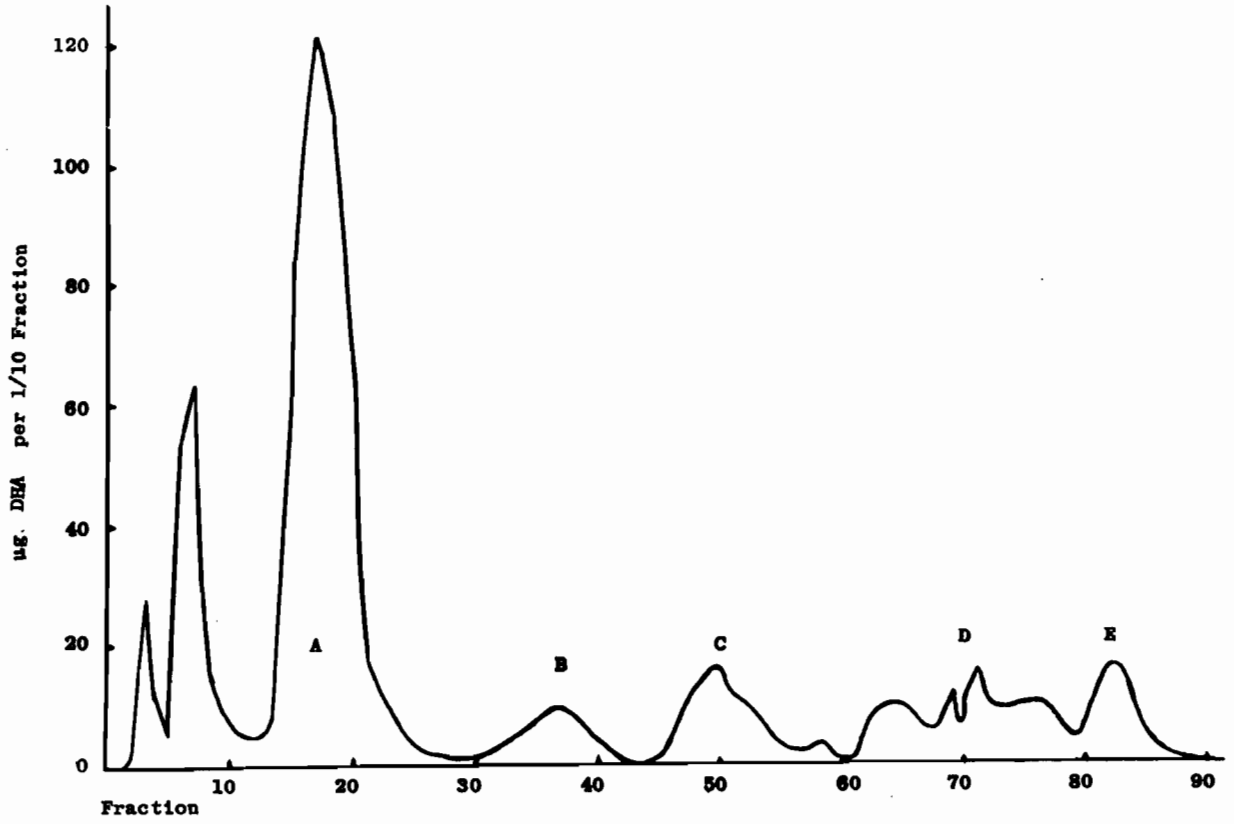
EXPERIMENTAL DESIGN

These studies were done with the assistance of Dr. J.I. Raeside at the Ontario Veterinary College at Guelph, Ontario. Urines were collected from the animals in metabolic cages, frozen and sent to Montreal.

In the preliminary studies, the urines were not collected on a 24-hour basis and only a qualitative picture of the urinary 17-ketosteroid excretion was obtained. Urines from normal males and females and gonadectomized males and females and pregnant females were examined in this way. Two liters of each urine specimen were processed. A sample pattern of the gradient elution chromatogram of the 17-ketosteroids from normal boar urine is shown in Figure XI. The first two peaks did not have typical Zimmerman chromogens and contained a great deal of pigments. For these reasons they were not further investigated. The peak eluted in Fractions 13 to 28 (Peak A) was first chromatographed on paper in ligroin C : propylene glycol. The Zimmerman-positive material having the polarity of dehydroisoandrosterone was eluted and chromatographed on paper in ligroin B : methanol :

Figure XI.

GRADIENT ELUTION CHROMATOGRAM OF 17-KETOSTEROIDS FROM NORMAL BOAR URINE



water (10 : 9 : 1). In this system two Zimmerman-positive areas were obtained, having the mobilities of dehydroisoandrosterone and isoandrosterone. These two areas were eluted and the residues were percolated through small alumina columns. The column eluates were then examined by infra red spectroscopy. The spectra were identical with those of dehydroisoandrosterone and isoandrosterone. Fractions 29 to 43 (Peak B) from the column were chromatographed on paper in the ligroin C : propylene glycol system and the Zimmerman-positive area with the mobility of androsterone was eluted. The residue was percolated through a small alumina column and submitted for infrared analysis. The spectrum was identical to that of our reference androsterone. Fractions 44 to 61 (Peak C) were treated similarly and the area corresponding to the etiocholanolone standard was eluted from paper. The infrared spectrum following the small alumina column in this instance was poorly defined so the material was rechromatographed on thin layer plates. A cleaner sample was obtained which gave an infrared spectrum identical to that of etiocholanolone. Fractions 62 to 79 (Area D) were pooled and chromatographed on paper in the toluene : propylene glycol system. Two Zimmerman positive areas were found, one with the mobility of 11—ketoetiocholanolone and one with the mobility of 11β-hydroxyandrosterone. The 11-ketoetiocholanolone area was chromatographed on a thin layer plate in the ethyl acetate : n-hexane (4:1) system, percolated through a small silica gel column and submitted for infrared analysis. The resulting spectrum was identical to that of 11β-hydroxyetiocholanolone. The urine extracts from the other classes of animals were treated in a similar manner.

The effects of ACTH and HCG on the urinary excretion of the individual 17 -ketosteroids were studied in a two-year-old boar of the Yorkshire-Wessex Saddleback crossbreed. For the first study, control 24-hour urines were collected followed by the intramuscular administration of 300 units per day of porcine ACTH for 8 days. The urine collected during the eight-day period was divided into two pools. Urine from days 1 to 4 and that from days 5 to 8 were pooled and 2 liter aliquots were removed for analysis. Two weeks later, a second control urine was obtained after which 2400 units per day of HCG were administered intramuscularly for 6 days. The urine was divided into two pools - days 1 to 4, and 5 and 6,- and two liter aliquots from the two were removed for analysis. The urine volumes varied from 4 to 9 liters / 24 hours during the two studies. Aliquots were removed from the various fractions after the paper chromatography for the quantitation of the individual 17—ketosteroids using the micro Zimmerman reaction as described in Part I.

RESULTS

The steroids isolated from the urines of the normal and gonadectomized male and female and pregnant female hogs are shown in Table XXIX. As can be seen, the 17-ketosteroids isolated from the urine of the normal boar are those normally found in human urine. In the preliminary studies, there was only chromatographic evidence for the presence of 11β-hydroxyandrosterone. In the latter studies it was identified by its infrared spectrum. Only the 11-oxy—17-

ketosteroids were found in the urine of the castrated boar. No 17-ketosteroids were detected in the urine of the normal female even though several large aliquots of urine were processed. The 11-oxy-17-ketosteroids were found in the urine from the ovariectomized sow. Androsterone and 11β-hydroxyandrosterone were positively identified in the urine of a pregnant sow and there was fair evidence for the presence of 11β-hydroxyetiocholanolone.

The gradient elution patterns of the urinary 17-ketosteroids of urine from a normal boar before and after stimulation with ACTH and HCG are shown in Figure XII. The excretion of the 17-ketosteroids was stimulated by HCG and depressed by ACTH. This increase is quite marked in the peak representing dehydroisoandrosterone plus isoandrosterone. The amounts of the individual 11-desoxy-17-ketosteroids of the urine pools collected during ACTH administration and during HCG administration are shown in Tables XXX and XXXI, respectively. The changes in the relative sizes of the dehydroisoandrosterone plus isoandrosterone peaks from the gradient elution columns are reflected in the changes in the quantities of both 17-ketosteroids as shown in the tables.

During ACTH administration the dehydroisoandrosterone excretion decreased from a control value of 41.5 mg per 24 hours to 10.2 mg per 24 hours during the sixth to eighth day of treatment. The corresponding figures for isoandrosterone were 27.2 and 3.2 mg per 24 hours, respectively. The androsterone excretion was lower than the control during the days 1 — 4 urine pool, but was the same as

TABLE XXIX

STEROIDS IDENTIFIED IN HOG URINE

Normal Boar	-	Dehydroisoandrosterone
		Isoandrosterone
		Androsterone
		Etiocholanolone
		11-Ketoetiocholanolone
		11β-hydroxyandrosterone
		11β-hydroxyetiocholanolone
Normal Sow	-	None
Castrated Boar	-	11-Ketoetiocholanolone
		11β-hydroxyandrosterone
		11β-hydroxyetiocholanolone
Ovariectomized Sow	-	11-Ketoetiocholanolone
		11β-hydroxyandrosterone
		11β-hydroxyetiocholanolone
Pregnant Sow	-	Androsterone
		11β-hydroxyandrosterone
		11β-hydroxyetiocholanolone ?

Figure XII.

EFFECT OF ACTH AND HCG ON THE ELUTION PATTERN OF URINARY 17-KETOSTEROIDS IN THE BOAR.

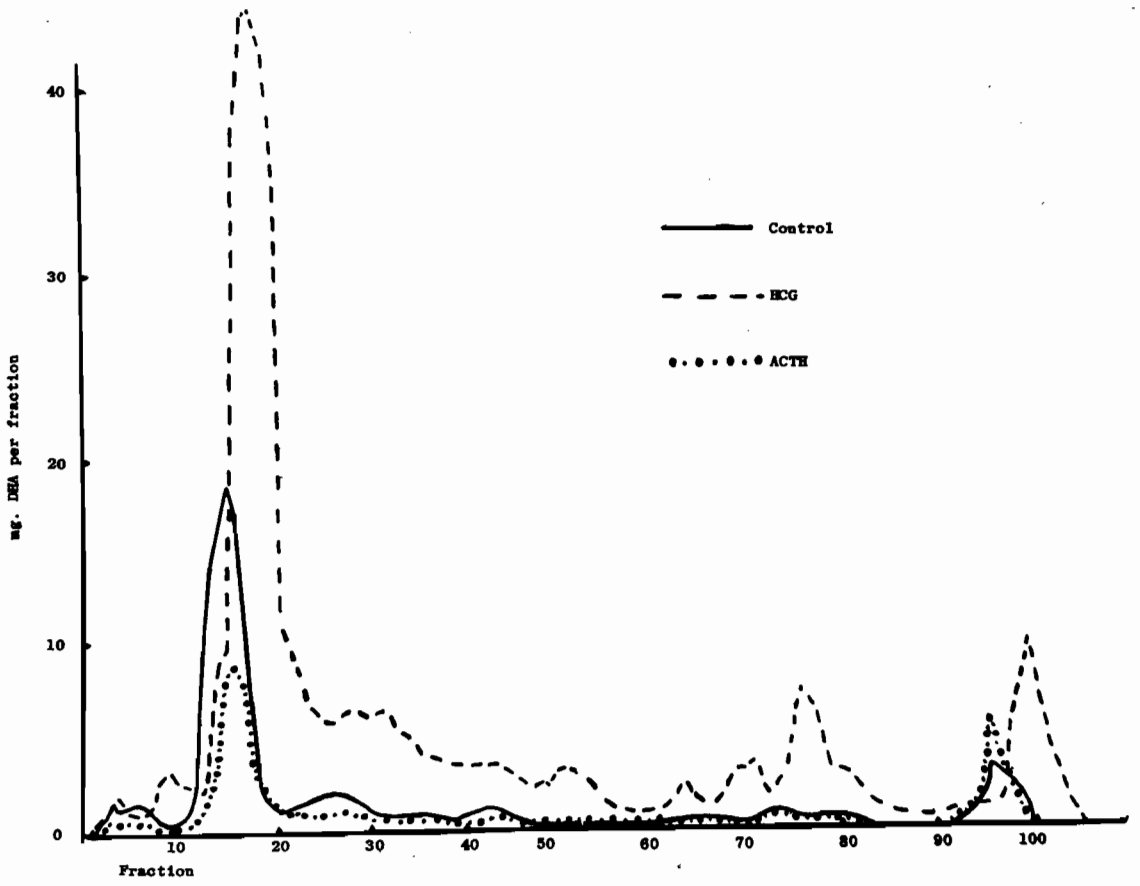


TABLE XXX

INFLUENCE OF HCG ON THE URINARY EXCRETION OF 17-KETOSTEROIDS BY THE BOAR

	Time Period		
	Control	Days 1-4	Days 5-6
		(mg/24 hours)	
Dehydroisoandrosterone + Isoandrosterone	76.7	239.9	198.2
Dehydroisoandrosterone (D)	41.7	143.6	100.9
Isoandrosterone (I)	33.2	100.6	56.9
D/I	1.26	1.43	1.77
Androsterone	0.43	14.72	15.2
Etiocholanolone	0.85	14.47	11.35

TABLE XXXI

INFLUENCE OF ACTH ON THE URINARY EXCRETION OF 17-KETOSTEROIDS BY THE BOAR

	Time Period		
	Control	Days 1-4	Days 6-8
		mg/24 hours	
Dehydroisoandrosterone + Isoandrosterone	70.4	33.2	13.6
Dehydroisoandrosterone (D)	41.5	21.4	10.2
Isoandrosterone (I)	27.2	9.0	3.2
D/I	1.53	2.38	3.18
Androsterone	3.6	1.31	3.61
Etiocholanolone	3.1	0.67	1.03

the control level during the days 6 — 8 pool. The urinary excretion of etiocholanolone was lower than the control value of 3.1 mg per 24 hours in both urine pools. The excretion of the four 17-ketosteroids quantitated, dehydroisoandrosterone, isoandrosterone, androsterone and etiocholanolone increased during HCG administration. There was a 3 - 4 fold increase in dehydroisoandrosterone and isoandrosterone excretion during days 5 and 6. Proportionately, there were even greater increases in the excretion of androsterone and etiocholanolone although in this study the control levels of excretion were lower than for the ACTH study (the same animal was used for both studies).

DISCUSSION

The urines of normal and castrated male hogs and of normal, ovariectomized and pregnant female hogs were examined for 17-ketosteroids. The urines were treated with β-glucuronidase and by solvolysis to hydrolyse the steroid conjugates. The neutral extracts were then chromatographed on alumina using the gradient elution technique. The various 17-ketosteroid fractions were then purified further by paper chromatography and percolation through small columns. The eluates from the small columns were then subjected to infrared analysis for positive identification of the individual 17-ketosteroids. In several instances, the 11-oxy-17-ketosteroids were identified as their acetates.

The excretion pattern of 17-ketosteroids by the normal boar is qualitatively similar to that found in the human (see Table XXIX). The 11-desoxy-17-ketosteroids, dehydroisoandrosterone, isoandrosterone, androsterone and etiocholanolone and the 11-oxy-17-ketosteroids, 11-

ketoetiocholanolone, 11β-hydroxyandrosterone and 11β-hydroxyetiocholanolone were present. The patterns of humans and hogs differ quantitatively as will be discussed later. The 11-desoxy-17-ketosteroids were absent in the urine of the castrated boar, although the 11-oxy-17-ketosteroids were present. This indicated that the testes were the source of all the 11-desoxy-17-ketosteroids in the urine of the normal boar. In man, the precursors of the urinary 11-desoxy-17-ketosteroids are largely elaborated by the adrenal glands.

It would appear from the results obtained with the boars and sows that the adrenals are the source of the 11-oxy-17-ketosteroids in both the males and females. The consistent absence of 17-ketosteroids in the urine of the normal sow was surprising. Two liter portions of two different urine collections were examined but no Zimmerman-positive substances were found in the gradient elution column eluates. In view of these results, the finding of 11-oxy-17-ketosteroids in the urine of the ovariectomized sow was unexpected. It is possible that with removal of the ovaries, a pituitary inhibitor was removed, with a consequent increase in the release of hormones which stimulate the adrenals. This might be an increase in ACTH secretion or an increase in gonadotrophic hormone production. Under normal conditions, the gonadotrophin secretion may not be high enough to significantly affect the adrenals.

Androsterone and 11β-hydroxyandrosterone were positively identified in the urine from the pregnant female. There was good chromatographic evidence for the presence of 11β-hydroxyetiocholanolone.

The Zimmerman-positive material had the mobility of 11β-hydroxyetiocholanolone in the toluene : propylene glycol system and after acetylation had the mobility of 11β-hydroxyetiocholanolone-3-acetate in the ligroin B : methanol : water (10 : 9 : 1) system. The infrared spectrum of the acetate contained all the major bands but some of the smaller bands in the fingerprint region were not well defined. The presence of androsterone suggests that in pregnancy either the placenta or fetus is producing 11-desoxy-17-ketosteroid precursors. One should also consider the possibility that the adrenals and/or the ovaries are being stimulated in a different manner during pregnancy.

The large increase in the excretion of 11-desoxy-17-ketosteroids following the administration of HCG to the normal boar is further support for the postulation that the testes are the sites of synthesis of the precursors of these steroids. The increases in the urinary levels of dehydroisoandrosterone and isoandrosterone during the HCG experiment were striking, both being excreted in excess of 100 mg per 24 hours during the first four days of the experiment. The lower levels found in the 6 to 8 day pool cannot be explained since the HCG was still being administered but it is possible that the testes could no longer be stimulated to the same degree or that antibodies to HCG were being formed. It is interesting to note that the ratio of dehydroisoandrosterone to isoandrosterone did not change significantly during the administration of HCG. Lindner (278) studied the effects of HCG on the secretion of androgens by boar testes by measuring the steroid levels in the spermatic vein blood. He did not find

dehydroisoandrosterone, but the levels of testosterone were quite significantly elevated. If the testes are secreting dehydroisoandrosterone as indicated by our studies, it is possible that it is secreted as the sulphate conjugate and so escaped detection in Lindner's studies. The urinary excretions of androsterone and etiocholanolone were also significantly increased during the administration of HCG. Whether these elevations are due to stimulation of dehydroisoandrosterone secretion or of testosterone secretion cannot be determined from these studies. If dehydroisoandrosterone is the main precursor of urinary androsterone or etiocholanolone, as is true in humans, then the efficiencies of these peripheral conversions must be low. The urinary excretions of these two metabolites is very low in comparison to the excretion of dehydroisoandrosterone. It appears that the metabolism of the dehydroisoandrosterone and perhaps testosterone favours the formation of isoandrosterone which is excreted in large amounts in the urine.

Dehydroisoandrosterone and isoandrosterone were significantly decreased by the administration of porcine ACTH. The urinary excretion of 17 -hydroxycorticosteroids increased from undetectable levels to 58.9 mg per 24 hours in the urine collected during the first 4 days of ACTH administration (assays performed by the Endocrine Laboratory, Royal Victoria Hospital). This indicated that the adrenals were being stimulated. If the testes are the source of the precursors of the urinary 11-desoxy—17-ketosteroids, then it is possible that the excessive secretion of adrenal steroids inhibits gonadal steroid

synthesis. During ACTH administration the ratio of dehydroisoandrosterone to isoandrosterone doubled, indicating that the suppression of isoandrosterone formation was greater than that of dehydroisoandrosterone formation.

These experiments indicate that the testes is the source of the major fraction of the 11-desoxy-17-ketosteroids excreted by the boar. It would also appear that the adrenals and gonads can influence one another, possibly through control of pituitary activity.

SUMMARY AND CONCLUSIONS

The conversion of 17α-hydroxypregnenolone-7α-H^3 to urinary dehydroisoandrosterone, androsterone and etiocholanolone was measured in a normal male subject and found to be 0.90 per cent. When the circulating pool of dehydroisoandrosterone was increased in the same subject by the oral ingestion of dehydroisoandrosterone, the conversion of 17α-hydroxypregnenolone to the three urinary 11-desoxy-17—ketosteroids was 0.99 per cent, thus indicating that the increased pool size had no significant effect on the total conversion, although the individual conversions were somewhat altered. These data provide additional evidence to support the view that metastatic tissue in patients with adrenal carcinoma is responsible for the enhanced conversion of 17α-hydroxypregnenolone to the urinary 11-desoxy-17-ketosteroids.

The conversions of simultaneously administered 17α-hydroxypregnenolone-7α-H^3 and 17α-hydroxyprogesterone-4-C^{14} to urinary pregnanetriol, 17α-hydroxypregnanolone and pregnenetriol were measured in a female subject with Cushing's syndrome due to bilateral adrenal hyperplasia. These conversions were studied both before and after bilateral adrenalectomy. The results obtained before and after bilateral adrenalectomy did not differ significantly indicating that the presence of the adrenals or their high levels of secretion did not affect the conversions measured. The ratio of H^3/C^{14} in the urinary pregnanetriol was 1/3 to 1/4 of the administered H^3/C^{14} ratio, indicating that the conversion of 17α-hydroxyprogesterone to pregnanetriol was more efficient than that of 17α-hydroxypregnenolone to the same metabolite. The H^3/C^{14} ratio in the

urinary 17α-hydroxypregnanolone was less than 1/20th. of the administered ratio. Pregnenetriol was quantitatively the most important metabolite of 17α-hydroxypregnenolone. These results indicate that 17α-hydroxy-pregnenolone is not always as important a precursor of urinary pregnanetriol as was suggested from the studies of others on subjects with adrenal neoplasms.

Urines from normal and gonadectomized male and female hogs and pregnant females were examined for their 17-ketosteroids. Dehydro-isoandrosterone, isoandrosterone, androsterone, etiocholanolone, 11-ketoetiocholanolone, 11β-hydroxyandrosterone, 11β-hydroxyetiocholanolone, were isolated from normal boar urine. The three 11-oxy-17-ketosteroids were found in the urine of castrated boars and ovariectomized sows. No 17-ketosteroids were found in the urine from normal sows. Androsterone and 11β-hydroxyandrosterone were isolated from the urine of pregnant sows. HCG administration resulted in a large increase in the daily excretion of the urinary 11-desoxy-17-ketosteroids by the boar. ACTH administration led to a decrease in the urinary 11-desoxy-17-ketosteroids in the normal boar. These results indicated that in the boar, the testes are the source of the 11-desoxy-17-ketosteroid precursors. In humans, the principal source of these precursors is the adrenals.

CLAIMS TO ORIGINAL RESEARCH

1. The total conversion of 17α-hydroxypregnenolone to urinary dehydroisoandrosterone, androsterone and etiocholanolone was not changed by increasing the circulating pool of dehydroisoandrosterone. These data provide additional evidence to support the view that metastatic tissue in patients with adrenocortical carcinoma is responsible for the enhanced conversion of 17α-hydroxypregnenolone to the urinary 11-desoxy-17-ketosteroids.

2. The conversions of simultaneously administered 17α-hydroxy-pregnenolone and 17α-hydroxyprogesterone to urinary pregnanetriol, 17α-hydroxypregnanolone and pregnenetriol were not affected significantly by the presence of the adrenals and their secretions in a subject with Cushing's syndrome due to bilateral adrenal hyperplasia. The conversion of 17α-hydroxyprogesterone to urinary pregnanetriol was 3 to 4 times more efficient than the conversion of 17α-hydroxypregnenolone to the same metabolite. Pregnenetriol was quantitatively the major metabolite of 17α-hydroxypregnenolone. The results indicate that 17α-hydroxy-pregnenolone is not always as important a precursor of urinary pregnanetriol as was suggested from the studies of others on subjects with adrenal neoplasms.

3. The male hog had a urinary 17-ketosteroid pattern qualitatively similar to that of humans. The castrated boar excreted only the 11-oxy-17-ketosteroids. The normal female hog excreted no 17-ketosteroids, while the ovariectomized sow excreted the 11-oxy-17-ketosteroids. The pregnant hog was found to excrete androsterone and 11α-hydroxyandrosterone.

Administration of HCG and ACTH resulted in an increase and a decrease, respectively, in the daily urinary excretion of 11-desoxy-17-ketosteroids by the normal boar. These results suggest that the testes are the source of the precursors of urinary 11-desoxy-17-ketosteroids. In humans the principal source of these precursors is the adrenals.

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