STUDIES ON THE FORMALDEHYDOGENIC SUBSTANCES

PRESENT IN HUMAN URINE.

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GENERAL INTRODUCTION.

In 1855 Addison described the disease which now bears his name and by post-mortem studies found that the adrenal glands were diseased. Publication of Addison's work stimulated Brown-Sequard (1856) to remove both adrenals from cats, dogs, rabbits and guinea-pigs. Death occurred within twelve hours, more rapidly than after removal of the kidneys, suggesting that the adrenal glands contained something which was essential to life. Removal of one adrenal alone was not sufficient to cause death. After a lapse of seventy years Rogoff and Stewart (1928) reported that the life span of adrenalectomized dogs could be prolonged by the administration of extracts of whole adrenals. These results were criticized by Swingle and Pfiffner (1931) who were the first investigators to demonstrate conclusively that a substance essential for the maintenance of life can be extracted with alcoholic benzene from the adrenal cortex. When a crude lipid fraction of the adrenal cortex was injected subcutaneously into bilaterally adrenalectomized cats the survival period of the cats was considerably prolonged.

Grollman/

Grollman and Firor (1932) found that a benzene extract of human urine also prolonged the life of adrenalectomized animals.

Between 1935 and 1938 twenty one crystalline steroids were isolated from the adrenal cortex by Kendall, by Reichstein and by Wintersteiner and Pfiffner and the physiological activities of many of these substances were studied. From 1942 to 1946 it was shown by Dorfman and by Venning that urine extracts possessed similar physiological activities and the group of substances responsible for the activity was termed the 'cortin' fraction. The chemical behaviour and biological activity of this fraction suggested that it consisted of a mixture of compounds similar to those isolated from the adrenal cortex. This was confirmed when a crystalline ketone which reduced alkaline silver and increased the storage of glycogen in the liver was isolated from post-operative urine by Venning, Hoffman and Browne (1944). Conclusive evidence was provided by Mason and Sprague (1948) who isolated cortisol (11β:17α:21-trihydroxy-pregn-4-ene-3:20-dione) from the urine of a boy with Cushing's syndrome. Prior to this isolation a steroid with an/

an oxygen atom at position 11, typical of the adrenocortical compounds, 11-ketoetiocholanolone (3α-hydroxy-5β-androstane-11:17-dione) had been obtained from normal urine by Lieberman, Dobriner, Hill, Fieser and Rhoads (1948). This is a possible metabolite of cortisol but could also arise in other ways. In 1950 Schneider isolated from normal human urine cortisone (17α:21-dihydroxy-pregn-4-ene-3:11:20-trione), tetrahydrocortisone (3α:17α:21-trihydroxy-5β-pregnane-11:20-dione) and three other crystalline compounds. Qualitatively and quantitatively the amount of cortisone isolated largely accounted for the activity of the cortin fraction in the liver glycogen assay.

Were it not for two fortunate facts the isolation of cortisol and cortisone from urine and our knowledge of corticoid metabolism might have been delayed for some time. Unlike the male sex hormone or the progestational hormone, which only occur in urine in special conditions, the active hormones themselves normally occur in urine. Testosterone has only been found in urine after its administration in large doses to humans and progesterone/

progesterone has only been detected in pregnancy urine. Also the metabolites of testosterone and progesterone appear in the urine in a conjugated form and the free steroids were liberated by acid hydrolysis. Such hydrolysis destroys corticoids with a hydroxyl group at position 17 so that it was fortunate that cortisol and cortisone appear in urine in the unconjugated state.

Fig. 1 shows the formula of a C-21 steroid. In the C-21 compounds the side chain has the β configuration at C-17 and if a hydroxyl group is present at C-17 this has the α configuration. In the C-19 steroids the side chain at C-17 is absent and replaced by a hydroxyl group which then occupies the β position or more often a ketone group is present at C-17.

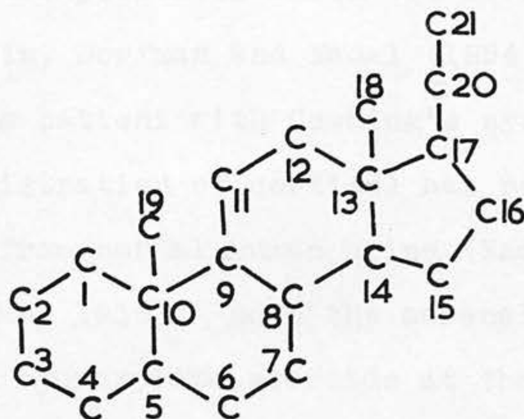


Fig. 1.

Dorfman and Ungar (1953) have recently compiled tables showing all the C-19 and C-21 steroids which have been isolated from natural sources but their tables have two defects: (1) no physical properties are recorded, and (2) the steroids listed in each table are not in any order. Tables 1 and 2[x] show respectively the C-19 and C-21 steroids listed in order of increasing oxygen content isolated from or identified in urine from humans whether normal, treated with adrenocorticotrophic hormone (ACTH) or with tumour or hyperplasia of the adrenal cortex. Compounds isolated after the administration of steroids are not included. Some of the compounds listed are artifacts produced during hydrolysis of conjugates.

6β-Hydroxycortisol which was first isolated by Burstein, Dorfman and Nadel (1954) from the urine of a patient with Cushing's syndrome after the administration of cortisol has recently been isolated from normal human urine (Nadel, Burstein and Dorfman, 1956). Both the adrenals and the liver can hydroxylate steroids at the 6β position but the significance of the reaction is not yet known/

x See Appendix II.

known. The 6β-hydroxy steroids synthesized by Ehrenstein and his co-workers had little activity. The two compounds 3α-hydroxy-5β-pregn-16-en-20-one and 3β-hydroxy-androst-5-ene-7:17-dione isolated by Fukushima, Kemp, Schneider, Stokem and Gallagher (1954) are possibly artifacts. The first could arise from a 16-hydroxylated compound and it is known that 5-ene steroids are capable of facile autoxidation at C-7 (Wintersteiner and Bergstrom, 1941). Other steroids isolated from urine since the monograph of Dorfman and Ungar are commented upon later.

Not the whole array of steroids found in urine are secreted as such by endocrine organs. Table 3 lists those steroids which have been isolated from or identified in blood. In humans cortisol is quantitatively the main secretory product. The amount of corticosterone present seems very variable; some investigators have not detected any, others have found an amount equal to that of cortisol. Aldosterone, present in peripheral blood in minute amounts, appears to be responsible for about half of the mineralocorticoid activity of human blood. The isolation by Farrell _et al_. of 11-deoxycorticosterone/

Table 3. Steroids which have been isolated from or identified in blood.

Authors	Source	Steroids identified
Reich, Nelson & Zaffaroni (1950). J.biol.Chem. 187, 411.	Dog adr.vein blood after ACTH	Cortisol Corticosterone
Bush (1951). J.Physiol. 112, 10P.	Dog adr. vein blood	Cortisol
Nelson, Samuels, Willardson & Tyler (1951). J.Clin.Endocrinol. Metab. 11, 1021.	Human peripheral blood	Cortisol
Pincus, Hechter & Zaffaroni (1951). Proc. Second Clinical ACTH Conference, 1, 40. The Blaikiston Co. New York	Cow peripheral blood	Cortisol Corticosterone 11-Deoxycorticosterone
Edgar (1952) Nature, 170, 543.	Ewe ovarian vein blood	Progesterone
Savard, Kolff & Corcoran (1952). Endocrinol. 50, 366.	Dog peripheral blood	Cortisol Corticosterone
Simpson, Tait & Bush (1952). Lancet, 2, 226.	Dog and monkey adr. vein blood	Aldosterone
West, Hollander, Kritchevsky & Dobriner (1952). J.clin.Endocrinol. Metab. 12, 915.	Dog spermatic vein blood	Testosterone Androst-4-ene-3:17-dione

Table 3 contd.

Authors	Source	Steroids identified
Bush & Sandberg (1953). J.biol. Chem. 205, 783.	Human plasma	Cortisol
Bush (1953). J. Endocrinol. 9, 95.	Adr. vein Blood: Rat	Cortisol, Cortico-sterone } Ratio (R) = 0.05; 11β-hydroxy-androst-4-ene-3:17-dione
	Rabbit	R = 0.1
	Ferret	R = 1.5
	Cat	R = 4.0; Cortisone; 11-Dehydrocorticosterone; 11β-Hydroxy-androst-4-ene-3:17-dione
	Monkey	R = 20
Farrell & Lamus (1953). Proc.Soc. Biol.Med. 84, 89.	Dog adr. vein blood	Cortisol Corticosterone 11-Deoxycortisol
Morris & Williams (1953). Biochem.J. 54, 470.	Human peripheral blood	Cortisol Cortisone Corticosterone 11-Dehydrocorticosterone
Pincus, Hudson & Romanoff (1953). J.clin. Endocrinol. Metab. 13, 1546.	Human adr. vein blood after ACTH	Cortisol Corticosterone Tetrahydrocortisol Tetrahydrocortisone Androst-4-ene-3:17-dione 11β-Hydroxy-androst-4-ene-3:17-dione
Zaffaroni & Burton (1953). Arch.Biochem. Biophys. 42, 1.	Dog adr.vein blood after ACTH	Cortisol Corticosterone

Bongiovanni/

Table 3 contd.

Authors	Source	Steroids identified
Bongiovanni, Eberlein, Grumback, van Wyk & Clayton. (1954). Proc. Soc. exp. Biol. Med. 87, 282.	Human plasma	Cortisol Tetrahydrocortisone
Farrell, Royce, Rauschkolb, & Hirschmann (1954). Proc. Soc. exp. Biol. Med. 87, 141.	Dog adr. vein blood	Aldosterone
Farrell, Rauschkolb, Royce & Hirschmann (1954). Proc. Soc. exp. Biol. Med. 87, 587.	Dog adr. vein blood	11-Deoxycorticosterone
Migeon & Plager (1954). J. biol. Chem. 209, 767.	Human peripheral plasma	Dehydroepiandrosterone Androsterone Etiocholanolone
Zander (1954). Nature, 174, 406.	Human peripheral blood (pregnancy)	Progesterone
Abelson, Ulrich & Long (1955). Proc. Soc. exp. Biol. Med. 89, 386.	Rat plasma after cortisol	20β-Hydroxycortisol
Bongiovanni & Eberlein (1955). Pediatrics, 16, 628. J. clin. Endocrinol. Metab. 15, 1531.	Human peripheral blood (adr. hyperplasia)	11-Deoxycortisol 3α:17α:21-trihydroxy-5β-pregnan-20-one
Clayton, Bongiovanni & Papadatos (1955). J. clin. Endocrinol. Metab. 15, 693.	Human plasma	Dehydroepiandrosterone Androsterone

Hudson/

Table 3 contd.

Authors	Source	Steroids identified
Hudson & Lombardo (1955). J.clin. Endocrinol.Metab. 15, 324.	Human adr.vein blood	Cortisol Corticosterone
Simpson & Tait (1955). Ciba Found.Colloquia on Endocrinol. 8, 204.	Human peripheral blood	Aldosterone
Sweat (1955). J. clin.Endocrinol. Metab. 15, 1043.	Human adr.vein blood	Cortisol Corticosterone
	Human peripheral blood	Cortisol
Neher & Wettstein (1955). Acta Endocrinol. 18,386.	Cow peripheral blood	Aldosterone
Bush, Swale & Patterson (1956). Biochem.J. 62, 16P.	Human adr.vein blood (virilism)	Cortisol Corticosterone 11β-Hydroxy-androst-4-ene-3:17-dione Dehydroepiandrosterone Androsterone
Migeon (1956). J. biol.Chem. 218,941.	Human peripheral plasma	Androsterone
Migeon, Sandberg, Bliss & Keller (1956). J. clin. Endocrinol. Metab. 16, 253.	Human plasma	Cortisol

sterone (21-hydroxypregn-4-ene-3:20-dione) proves that this steroid can be produced _in vivo_ by the adrenal. Although originally isolated from the adrenal in small amounts by Reichstein and von Euw (1938) it had subsequently only been detected on paper chromatograms of an adrenal extract and in perfusion experiments. It seems to be of little importance for it is present in very small amounts. More recently the tetrahydro-derivative of 11-deoxycorticosterone has been isolated from the urine of a pseudohermaphrodite with adrenal hyperplasia and hypertension by Eberlein and Bongiovanni (1955). These investigators have also isolated 11-deoxycortisol (17α:21-dihydroxy-pregn-4-ene-3:20-dione) and its tetrahydro-derivative from the blood and urine of the same patient. The tetrahydro-compound which had previously been isolated from the urine of patients with adrenal carcinoma has now been isolated from normal urine (Touchstone, Bulashenko, Richardson and Dohan, 1955). Thus 11-deoxycortisol which has been isolated from dog adrenal vein blood may be a normal secretory product of the human adrenal.

Several unidentified spots which give colour reactions/

reactions typical of the steroids have been found on chromatograms of adrenal vein blood and it is known from _in vitro_ work that the adrenal is capable of producing a large number of steroids. The nature and activity of these other components must await their isolation and certain identification. Many of the corticoids recently found in urine and blood have been identified only by their chromatographic properties and reactions and/or their infra-red spectrum.

A recent important discovery has been the detection of steroid conjugates in human blood. Migeon and Plager (1954) could isolate dehydro_epi_androsterone from human plasma after acid hydrolysis but not after glucuronidase hydrolysis. Bongiovanni (1954, Bongiovanni _et al_., 1954) found that after incubating serum, which had previously been extracted to remove free steroids, with a glucuronidase preparation more corticoids could be extracted. The steroid behaved chromatographically like tetrahydrocortisone and approximately equal amounts of free and conjugated steroid were present. Thus the conjugated fraction must also be taken into account when determining plasma steroid levels.

From/

From the presence of certain steroids in urine and from the increased excretion of some of the compounds after administration of various steroids it is possible to deduce the reactions that occur in steroid metabolism. These are summarized briefly below:-

(1) Ketone groups at positions 3 and 20 can be reduced to give both α and β isomers although the α appears to be the favoured position. Only compounds containing β-hydroxyl groups have resulted from <u>in vivo</u> reduction of ketone groups in the 11 and 17 positions. However, 5β-androstane-3α:17α-diol has been isolated from ox bile by Pearlman (1948) and 17α-hydroxy compounds have been isolated from <u>in vitro</u> reactions.

(2) Hydroxyl groups can be introduced at positions 17α and 21 in the C-21 steroids and at position 11β. Hydroxyl groups are also found at positions 6 and 16 in both configurations; whether these hydroxyl groups are introduced directly into the molecule as appears to be the case with 11β-hydroxylation or whether they arise by reduction of ketone groups is unknown. Dorfman (1955) has reported that 16-keto-oestrone

3-/

(3-hydroxy-oestra-1:3:5-triene-16:17-dione) has been isolated from normal female urine by Serchi (1953) and it has been shown that after administration to a normal male 16-keto-oestrone is reduced to oestriol. 6-Keto-progesterone (pregn-4-ene-3:6:20-trione) has recently been isolated from perfusates of human placentae by Hagopian, Pincus, Carlo and Romanoff (1956). Hydroxyl groups may also occur at positions 2, 18 and 19.

(3) Hydroxyl groups at positions 3, 11 and, in C-19 steroids, at position 17 can be oxidized to ketone groups.

(4) Double bonds at positions 1, 4 and 16 can be saturated and compounds of both the 5α and 5β series can be formed.

(5) The 17- and 21-hydroxyl groups in C-21 steroids can be removed forming 17- and 21-deoxy compounds. Since Marker and Lawson (1938) have isolated 5β-pregnan-3α-ol from pregnancy urine it is possible that the 20-hydroxyl group can also be removed.

(6) The 5-en-3β-ol group can be converted to the 4-en-3-one group.

(7) /

(7) The side chain of those steroids with a hydroxyl group at C-17 and a ketone group at C-20 can be removed forming a 17-ketosteroid.

It is estimated that the adrenal secretes between 15 mg. and 25 mg. of cortisol per day. It is known that this amount of cortisol or cortisone will maintain adrenalectomized patients. Dorfman (1954) estimated that 21 mg. per day of cortisol is secreted by the adrenal on the basis of the quantities of 11-oxy- and 11-deoxy-17-ketosteroids found in normal urine and the same value is suggested by Silber (1955) from a comparison of the amount of phenylhydrazine-reacting substances in extracts of normal urine and in extracts of urine from persons injected with cortisone. Bondy and Altrock (1953) using an inaccurate method estimated the amount secreted to be between 15 mg. and 25 mg. per day. Recently Peterson and Wyngarden (1956) using cortisol-4-C^{14} have found the turnover rate of endogenously synthesized cortisol to vary from 17 mg. to 29 mg. per day and the average value for the subjects studied was 22 mg. per day.

If these estimates are correct then we are now in a position to account quantitatively for the cortisol/

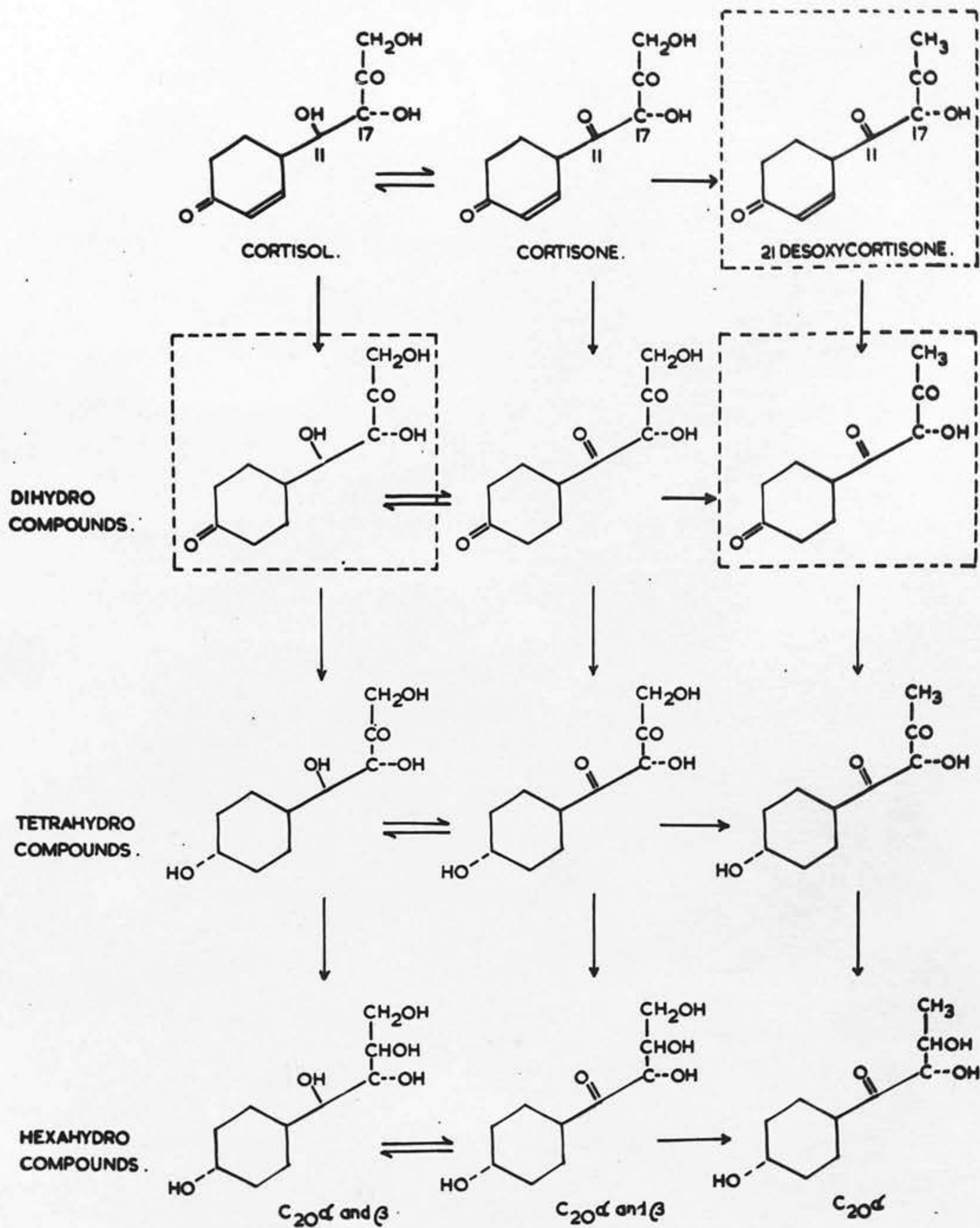


FIG.2. METABOLISM OF CORTISOL.

cortisol secreted by the adrenal as urinary metabolites. After the administration of cortisol labelled with C^{14} to humans it has been shown that more than 80% of the radio-activity appears in urine during the first 24 hours and that after three days 95% of the administered dose is recovered from urine (Peterson and Wyngarden, 1954). Fig. 2 depicts the possible metabolites of cortisol. For simplicity an abbreviated structural formula is used. The main metabolic reaction is reduction; thus one of the principal metabolites of cortisol is tetrahydrocortisone and Baggett, Kinsella and Doisy (1953) have found the daily excretion of this compound to vary from 2 mg. to 7.4 mg. Following this reduction there is probably competition between further metabolism and conjugation with glucuronic acid and excretion. Reduction of the 20-ketone group produces both epimers - the α and β cortols and cortolones. These are also excreted as conjugates of glucuronic acid and the four compounds account for at least 30% of the metabolites of administered cortisol (Fukushima, Leeds, Bradlow Kritchevsky, Stokem and Gallagher, 1955). Dihydrocortisone/

cortisone (17α:21-dihydroxy-5β-pregnane-3:11:20-trione) and tetrahydrocortisol (3α:11β:17α:21-tetrahydroxy-5β-pregnan-20-one) are also found in urine and have been estimated by Cope and Hurlock (1953). Values quoted for the excretion of the above compounds must be minimal ones since in all cases the estimation was performed after enzymic hydrolysis which may not be complete and the compounds also have an appreciable water solubility which causes losses during the extraction procedures.

Zondek and Finkelstein (1951, 1952, 1954) have found in urine a highly fluorogenic compound which has been shown to be the 21-deoxy derivative of tetrahydrocortisone (3α:17α:20α-trihydroxy-5β-pregnan-11-one) by Finkelstein, von Euw and Reichstein (1953). The amount excreted by normal humans, measured after acid hydrolysis, varied from 0.75 mg. to 3 mg. per day. Cortisol and cortisone have long been known to occur in urine in small amounts, approximately 20 μg. to 100 μg. of each daily. From experiments in which 17-hydroxy-corticosteroids have been administered to adrenalectomized humans and by the use of radio-active steroids it appears that about 5% of/

of cortisol or cortisone may be degraded to 17-ketosteroids. Rosselet, Jailer and Lieberman (1954) have demonstrated that the 17α-hydroxyl group of a C-21 steroid can be removed <u>in vivo</u>; after administration of 17α-hydroxypregn-4-ene-3:20-dione or 11β:17α-dihydroxy-pregn-4-ene-3:20-dione to adrenalectomized humans the 17-deoxy derivatives were isolated in 0.2% and 1% yield respectively. Nadel, Burstein and Dorfman (1956) have found that 0.6% of fed cortisol is hydroxylated at the 6β position. Table 4 summarizes the quantities of these metabolites found in normal urine; approximately 85% of the estimated production of cortisol can be accounted for agreeing well with the results of experiments with labelled cortisol.

Table 4 /

Table 4. Urinary excretion of cortisol metabolites; mg. per day.

Tetrahydrocortisone	2-7.4
Cortols and cortolones	approx. 8
Dihydrocortisone	0.2-0.7
Cortisol and cortisone	0.05-0.2
3α:17α:20α-trihydroxy-5β-pregnane-11-one	0.75-3
Conversion to C-19 steroids	1-1.5
Minor metabolic routes:- (a) 6β-hydroxylation (b) 17-deoxy derivatives (c) Tetrahydrocortisol	at least 0.5

Total daily excretion 12.5-21.3 mg.

Estimated daily production of cortisol 15-25 mg.

In Fig. 2 those steroids surrounded by a dotted line have not yet been isolated from urine. 3α:17α-Dihydroxy-5β-pregnane-11:20-dione has been isolated only from the urine of a patient treated with ACTH (Lieberman, Katzenellenbogen, Schneider, Studer and Dobriner, 1953).

The corticosterone secreted by the adrenal, unlike cortisol, cannot be accounted for as urinary metabolites. This is possibly due in part to the variable amounts of corticosterone secreted. Table 5 shows the metabolites of corticosterone/

Table 5. Metabolites of corticosterone isolated from or identified in urine.

Author	Conditions	Metabolites
Mason (1948). J.biol.Chem. 172, 783.	Addison's disease after 11-dehydrocorticosterone	3α:20α-dihydroxy-5β-pregnan-11-one
Lieberman, Fukushima & Dobriner (1950) J.biol.Chem. 182, 299.	Adrenogenital syndrome	3α-hydroxy-5β-pregnane-11:20-dione
	Normal	3α:20α-dihydroxy-5β-pregnan-11-one
Fukushima, Kemp, Schneider, Stokem & Gallagher (1954). J.biol.Chem. 210, 129.	After corticosterone	5β-pregnane-3α:11β:20α-triol 3α:11β-dihydroxy-5β-pregnan-20-one
Touchstone, Richardson, Bulashenko, Landolt & Dohan (1954). Arch.Biochem.& Biophys. 52, 284.	After ACTH	Corticosterone Tetrahydrocorticosterone allo-Tetrahydrocorticosterone
Engel, Carter & Fielding (1955). J.biol.Chem. 213, 99.	After corticosterone	Tetrahydrocorticosterone allo-Tetrahydrocorticosterone 3α:21-dihydroxy-5β-pregnane-11:20-dione
Dohan, Touchstone & Richardson (1955). J.Clin.Invest. 34, 485.	After ACTH	Corticosterone 11-dehydrocorticosterone allo-Tetrahydrocorticosterone 3α:21-dihydroxy-5β-pregnane-11:20-dione
Dyrenfurth & Venning (1955). Rev. Canad.Biol. 14, 249.	Normal	Corticosterone

corticosterone or 11-dehydrocorticosterone that have been isolated from or identified in urine. Only one compound, 3α:20α-dihydroxy-5β-pregnan-11-one, has been isolated from the urine of normal persons and this compound has not been found after administration of corticosterone or ACTH. Dyrenfurth and Venning (1955) have recently reported that they have detected corticosterone on chromatograms of extracts of normal urine. As with the 17-hydroxycorticosteroids reduction of ring A would appear to be a major metabolic reaction of the 17-deoxycorticosteroids and the tetrahydro derivatives of the 17-deoxycorticosteroids may be found in the urine of untreated persons. However, it may be that the 17-deoxycorticosteroids undergo reduction at C-21 forming C21-deoxy compounds at a faster rate than the 17-hydroxycorticosteroids. Then under normal conditions when relatively small amounts of corticosterone are secreted the tetrahydro compounds of corticosterone end 11-dehydrocorticosterone are predominantly reduced at C-21 whereas after administration of ACTH or corticosterone the increased amount of corticosterone is excreted mainly as the tetrahydro derivative. Compounds reduced at C-20 and/or C-21 would not have been detected/

detected in the experiments of Dohan _et_ _al_. since these workers were interested only in α-ketolic metabolites, nor would they be detected on paper chromatograms sprayed with the usual reagents. There is no evidence for the removal of an oxygen atom at position 11 or for the conversion of 17-deoxy compounds to 17-ketosteroids. It has been shown by Sandberg, Migeon and Samuels (1955) that after the intravenous injection of corticosterone-4-C^{14} to normal humans approximately 75% of the radio-activity appeared in the urine in 48 hours.

Although during the past eight years we have learnt much about the metabolism of the steroids secreted by the adrenal cortex their mode of action is still unknown and little is known concerning possible interaction of the hormones. Hechter (1955) recently reviewed the evidence suggesting that the cortisol secreted by the adrenal is changed into a more active form. Since then Wilson, Fairbanks, McEwen and Ziff (1955) have confirmed that cortisol is active when injected intra-articularly whilst cortisone is not. Paper chromatography showed that a number of metabolites were produced and they suggest that cortisol was active because it was transformed to a specific metabolic product.

PART I. A STUDY OF FORMALDEHYDOGENIC SUBSTANCES IN, AND THE ISOLATION OF CRYSTALLINE COMPOUNDS FROM, EXTRACTS OF BOILED URINE.

1. <u>Introduction</u>

In 1951 Speirs and Meyer developed a bioassay, 'specific for those corticosteroids with an oxygen atom at position 11 and a double bond at position 4', based upon a decrease in the number of circulating eosinophils in adrenalectomized mice after injection of the assay material. Later Speirs, Wragg, Bonner and Homburger (1951) found that boiling urine at a neutral or slightly alkaline pH without loss of volume increased its activity compared with the unboiled urine. Boiling under acid conditions (pH 2) destroyed the activity. Marrian and Atherden (unpublished) showed that if urine which had previously been extracted with chloroform was boiled for half an hour the amount of formaldehydogenic substances (FSS)[x] which could be/

x Those substances which give rise to formaldehyde on periodate oxidation. Of the steroids only those with an α-ketol or α-glycol grouping so react. The determination of FSS is described in Part II.

be extracted by chloroform from the boiled urine was approximately equal to that extracted from unboiled urine. Colàs (1955) showed by paper chromatography that extracts of boiled urine contained substances which would reduce blue tetrazolium and which were not present in extracts of unboiled urine. The substance present in largest amount was designated X and a second substance which was slightly more polar than X was termed Y. As described later X was transformed by acid into a compound termed Xa. From extracts of boiled urine Colàs isolated in crystalline form a ketonic compound designated K2.

Sections 3-7 of this part of the thesis describe experiments carried out in collaboration with Dr A. Colàs. Section 8 describes briefly the results obtained by Colàs and summarizes the information concerning the substances under investigation which had been collected at the time that Colàs completed his investigations. The remaining sections describe the further experiments performed by the candidate.

2. /

2. Methods

(a) Extraction of urine.

For the experiments described in Sections 3 to 7 normal male urine, collected without preservative, was extracted with 2 vol. chloroform. The extracted urine was then boiled for 1 hr., cooled and reextracted with 2 vol. chloroform. The chloroform extracts were washed twice with 1/5 vol. 0.1 N-sodium hydroxide solution, twice with 1/5 vol. distilled water, dried over anhydrous sodium sulphate and evaporated to dryness *in vacuo* at a temperature about 60°C.

Two procedures have been used for extracting the larger amounts of urine needed for isolation work. In neither of them was the urine extracted before boiling. In the first the urine after having been boiled was extracted 4 times with 1/10 vol. chloroform and any emulsions formed were broken by centrifugation. In the second the urine was concentrated to 1/10 of its original volume in a metal still, boiled for 1 hr. and extracted three times with a volume of chloroform equal to 1/20 of the original volume of urine. The chloroform extracts were then treated as described above.

(b) /

(b) Partition Procedure.

Urine extracts which were to be chromatographed were purified by the following procedure. The crude residue left after evaporation of the chloroform was dissolved in 10 ml. of 70% aqueous methanol (v/v) and the solution transferred to a small separating funnel containing 20 ml. hexane. The flask which had contained the urine residue was washed with another 10 ml. aqueous methanol and this was also transferred to the separating funnel. After shaking and equilibration the aqueous layer was removed. The hexane was re-extracted with 20 ml. aqueous methanol and the combined methanol extracts evaporated *in vacuo* until about 10 ml. of liquid remained. This was diluted with 10 ml. water and the solution extracted with 50 ml. of ether. The ether extract was dried over anhydrous sodium sulphate, filtered and evaporated to dryness *in vacuo*. The residue was transferred to a small tube and dissolved in methanol for chromatography.

The above volumes are suitable for residues weighing not more than 25 mg.

(c) Paper Chromatography.

The solvent systems developed by Bush (1952) listed below have been employed. These were used at/

at 18°C. and were satisfactory without modification except for system B5.

System A Light petroleum, methanol, water (10:8:2)

B1 Light petroleum, toluene, methanol, water (5:5:7:3)

B2 Light petroleum, toluene, methanol, water (3.3:6.6:6:4)

B5 Benzene, methanol, water (10:7:3) (modified)

Solvent mixtures were renewed monthly.

Whatman No. 42 chromatography paper was used. The material to be chromatographed was applied to the paper by means of a 0.1 ml. blood pipette calibrated in microlitres and with the tip drawn out into a fine capillary. The paper was held over a warm boiling plate and a measured amount of a methanolic solution of the residue to be chromatographed applied to the paper so that the resulting spot was less than 1 cm. in diameter. Development was allowed to take place until the solvent front was 8-10 inches from the starting line when the paper was removed from the tank and allowed to dry in air.

15 μg. amounts of pure steroids (either cortisol, cortisone or 11-deoxycortisol) were run as standards on each paper.

Compounds containing an αβ unsaturated ketone group/

group were detected by the fluorescence reaction of Bush (1952) or by their adsorption of ultraviolet light of wavelength 253.7 mμ. α-Ketolic compounds were detected by their ability to reduce an alkaline blue tetrazolium (BT) solution (10 mg. BT dissolved in 10 ml. water; to this solution 5 ml. of 10% sodium hydroxide solution are added).

(d) Column Chromatography of Urine Extracts.

For the isolation of K2 the extract from the ether phase of the partition procedure was chromatographed on 60 g. of a 1:1 mixture of magnesium silicate and Celite (Schneider, 1950a,b). This was made into a slurry with petroleum ether (boiling range 80-100°C.) and packed to form a column approximately 30 x 2.5 cm. The extract to be chromatographed, being insoluble in petroleum ether, was adsorbed onto a small amount of the magnesium silicate-Celite mixture which was packed onto the top of the column. Elution was performed by a modification of the gradient elution technique (Laksmannan and Lieberman, 1954) best described as discontinuous gradient elution. From a reservoir containing 1 litre of petroleum ether, 10 ml. were removed and poured onto the top of the column. Then 2 ml. of ethanol were added to the reservoir, the mixture/

mixture shaken and 10 ml. of this mixture next applied to the column. This process of removing 10 ml. of liquid from the reservoir and adding 2 ml. of ethanol was continued until 20 fractions of 40 ml. each had been collected. The rate of flow of eluate from the column was about 3 ml. per min. achieved by the application of positive pressure (about 6 cm. mercury) to the top of the column. The solvent was removed from each fraction by evaporation under a blast of air. An aliquot usually 1/200 of each alternate fraction was then chromatographed on paper in system B2 and those fractions shown to contain X were combined. The residue containing X was then subjected to a Girard separation.

The non-ketonic fraction resulting from the Girard separation and those fractions not containing any X were combined and stored. The ketonic fraction obtained from the 2nd, 3rd and 4th pools of urine was then chromatographed on 10 g. of a 1:1 mixture of magnesium silicate-Celite forming an 18 x 1 cm. column. The ketonic fraction was applied adsorbed on a small amount of the mixture and discontinuous gradient elution again used starting with 200 ml. petroleum ether in the reservoir, removing 10 ml. and adding 0.5 ml.

ethanol/

ethanol etc. 20 fractions each of 10 ml. were collected. An aliquot, usually 1/150 of the residue obtained from each alternate fraction, was chromatographed on paper in system B2 and those fractions shown to contain Xa were combined. In later work this second chromatography on magnesium silicate-Celite was omitted.

The residue containing Xa was then subjected to partition chromatography using Celite as the supporting medium. The stationary phase was 80% aqueous methanol and the mobile phase was benzene. The phases were mutually saturated and the columns were prepared as described by Butt, Morris, Morris and Williams (1951) and Bauld (1955). Sixty grams of Celite mixed with 60 ml. of stationary phase were packed to produce a column 32 x 2 cm. The residue containing Xa was dissolved in the smallest amount of stationary phase (usually 0.5-1.0 ml.), the required amount of Celite added to adsorb the solution and the Celite packed onto the top of the column. Eighteen fractions of 25 ml. were collected, the flow rate being 40 ml. per hr. After removal of the solvent 1/150 of alternate fractions were chromatographed on paper and those fractions shown to contain Xa were/

were combined, the residue dissolved in methanol and filtered, to remove traces of Celite, into a small (10-15 ml.) conical flask for crystallization.

The average characteristics of the columns used are described below. The average weights of the fractions at various stages, and index of the purification achieved, are also shown assuming the volume of urine extracted to be 200 litres.

Butt, Morris, Morris and Williams (1951) by modifying the general equation of Martin and Synge (1941) have derived a term V_r called the 'retention' volume. This is the volume of eluate collected before the peak concentration of the solute emerges from the column. If the partition coefficient of the solute is zero the retention volume for this solute is equal to the dead volume V_D of the column. These terms are useful for describing the characteristics of a column and the behaviour of a solute passing through the column. Since some of the pigments present in urine extracts usually have partition coefficients close to zero the dead volume recorded below denotes the eluate volume at which the front of the first pigment band appeared. Also since it is often difficult to determine when the peak concentration of any solute is leaving the column the/

the term V_r used below denotes the volume of eluate collected before the first fraction containing any of the solute under consideration is collected.

Volume of urine extracted 200 litres

Wt. of residue from ether phase of partition procedure 1.2-1.5 g.

1st Column: 60 g. magnesium silicate-Celite

V_D 180 ml. V_r 250 ml.

Wt. of X fraction 500-600 mg.

Wt. of ketonic fraction 160 mg.

Wt. of non-ketonic fraction 250-300 mg.

2nd Column: 10 g. magnesium silicate-Celite

V_D 40 ml. V_r 80 ml.

Wt. of Xa fraction 75 mg.

3rd Column: 60 g. Celite

V_D 45 ml. V_r 150 ml.

Wt. of Xa fraction 40-60 mg.

(e) Girard separation

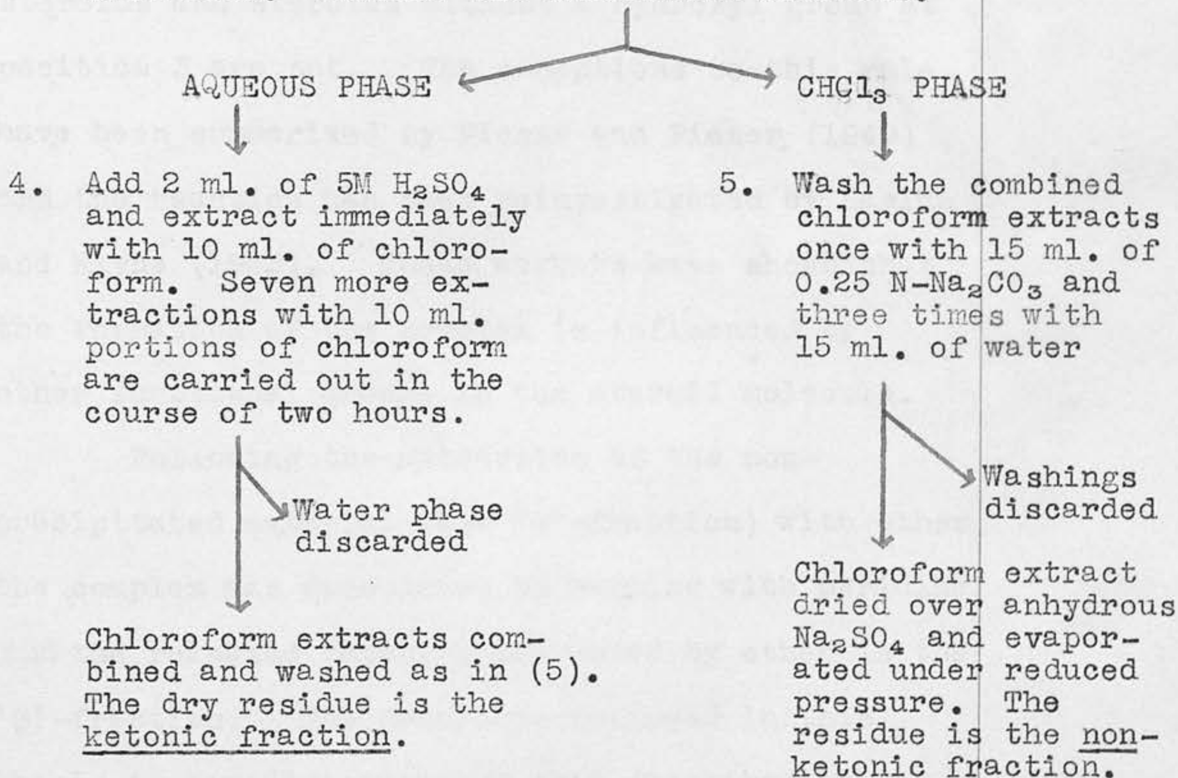
To separate those compounds containing a ketone group from those not containing one a modification of the method first introduced by Girard and Sandulesco (1936) was used. The procedure is shown in Fig. 3 and is essentially the same as that described by Schneider(1952) for the isolation of corticosteroids from human urine.

Fig. 3. Girard Separation.

1. Dissolve the crude fraction containing no more than 10 mg. of ketosteroids in 1 ml. of 50% (v/v) acetic acid in 90% (v/v) aqueous methanol. Add 100 mg. of Girard's reagent T and warm gently if necessary until complete solution. Leave the reaction mixture overnight at room temperature.

2. In the morning, add small pieces of ice and 10 ml. of ice-cold water. Transfer to a 50 ml. separating funnel. Add 3 ml. of ice-cold 2.5 N-NaOH. The flask is washed with 10 ml. of ice-cold water which are also transferred to the funnel.

3. Extract eight times with 10 ml. of ice-cold chloroform, allowing the separation of the two phases to take place each time. Combine the chloroform extracts.



(f) Digitonin precipitation.

For the separation of mixtures of steroids and the structural analysis of unknown steroids many applications have been made of the discovery by Windaus (1909) that cholesterol will form a complex with digitonin that is insoluble in alcohol. Fernholz (1935) found that most 3β-hydroxysteroids are precipitated by digitonin whilst most 3α-hydroxysteroids and steroids without a hydroxyl group at position 3 are not. The exceptions to this rule have been summarized by Fieser and Fieser (1949) and the reaction has been reinvestigated by Haslam and Klyne (1953). These workers have shown that the formation of the complex is influenced by other functional groups in the steroid molecule.

Following the extraction of the non-precipitated material (the 'α'-fraction) with ether, the complex was decomposed by warming with pyridine and the released material extracted by ether is the 'β'-fraction. The procedure followed in this thesis is a modification of that described by Butt, Henly and Morris (1948) and is shown in Fig. 4. It is known that some 3β-hydroxysteroids form soluble digitonides which are removed in the

α-/

Fig. 4. Digitonide Formation

1. The material, containing no more than 0.5 mg. of 3β-hydroxysteroids, is transferred to a graduated glass-stoppered centrifuge tube and dried. Add 0.75 ml. of a 1% digitonin solution in 90% aqueous ethanol warmed at 37°C. Fit a microcondenser to the tube and reflux gently for 30 min. The solvent is removed carefully under reduced pressure.

2. 10 ml. of ether are added and the dry residue dispersed with the aid of a thin glass rod. Centrifuge for 5 min. at 2000 r.p.m. Decant the supernatant into a 30 ml. separating funnel. Repeat this step with three 5 ml. portions of ether.

→ SOLID RESIDUE → step 4

→ SUPERNATANT AND WASHINGS → step 3

3. Wash the combined ether supernatants three times with 5 ml. portions of dist. water. Dry with a pinch of anhydrous sodium sulphate and evaporate. The residue is the

α-Fraction

4. The residue is dissolved in 0.25 ml. of pyridine, warmed to 60°C. for 3 min., cooled and 5 ml. of ether are added with stirring. Centrifuge, decant the supernatant into another 30 ml. funnel and repeat. The residue is further washed with 3 x 5 ml. of ether.

5. The combined ether supernatants are washed twice with 5 ml. portions of 2N-sulphuric acid and three times with 5 ml. portions of dist. water. Dry with a pinch of anhydrous sodium sulphate and evaporate. The residue is the

β-Fraction

α-fraction by the ether added to the alcoholic medium. To overcome this, in the procedure described in Fig. 4, the alcohol was removed by evaporation *in vacuo* after the formation of the complex.

(g) Crystallization of residues from urine extracts.

Methanol has been the solvent most usually employed for the crystallization of compounds in this thesis. Crystallizations using methanol were performed at -20°C. When the weight of material to be crystallized exceeded about 20 mg. or the volume of liquid used for crystallization exceeded 0.5 ml. the crystals were separated from the mother liquors by employing a Quickfit semi-micro Willstätter filter assembly. The filter assembly and solvent for washing the crystals were cooled before use. For the removal of mother liquors of less than 0.5 ml. and washing the crystals and removal of the washing solvent the above filter assembly is of little use. A modification of the Skau centrifuge tube (Cheronis, 1954) has been used and found to be satisfactory. The most satisfactory method for removing the mother liquors when these were less than 0.5 ml. in/

in volume has involved the use of a piece of pipe-clay. The crystallization of the material took place in a small glass tube (5 x 1.3 cm.) at -20°C. When crystallization was complete a piece of pipe-clay (approximately 7 x 0.6 cm.), previously chilled to -20°C., was placed in the small tube and the whole placed inside a stoppered boiling tube and left at -20°C. The pipeclay absorbed the supernatant liquid leaving the crystals in the small tube. The time necessary for absorption of the mother liquors varied from 1½ to 3 hr. The crystals were then dried *in vacuo* and weighed.

The pieces of pipeclay were cleaned by refluxing with chloroform and methanol and then heating to redness.

(h) Colour reactions.

Blue tetrazolium reaction for α-ketolic steroids.

The method of Chen, Wheeler and Tewell(1953) has been used. Improvements of this method have been described by Mader and Buck (1952) and Morris and Williams (1955).

To the steroid contained in a glass-stoppered test tube 1 ml. of methanol, 1 ml. of a 1N solution of NaOH in 80% aqueous methanol and 1 ml. of a 1% methanolic solution of blue tetrazolium (BT) were added/

added and the mixture allowed to stand in the dark in a water bath at 25± 2°C. for 20 min. The reaction was stopped by the addition of 4 ml. of methanol-acid pyridine (3 parts of methanol to 1 part of acid pyridine prepared by adding 20 ml. conc. hydrochloric acid to 100 ml. pyridine) and the solution stirred. The intensity of absorption of light of wavelength 520 mμ was measured using a Hilger Spekker photoelectric absorptiometer.

Pettenkofer reaction.

The method described by Munson, Jones, McCall and Gallagher (1948) has been used. The reaction is stated by the authors to be specific for steroids with unsaturation in ring B, or a group which can give rise to unsaturation in ring B, and a hydroxyl group or unsaturation in ring A. However the intensity of the colour produced in the reaction is also influenced by the presence of other functional groups in the molecule.

To between 10 and 50 μg. of the steroid contained in a test tube 0.5 ml. of glacial acetic acid was added followed by 2 ml. of furfural solution (0.56% v/v in 50% acetic acid) and 7.5 ml. of 8M sulphuric acid. After stirring, the tube/

tube was placed in a water-bath maintained at 67± 0.2°C. for 12 min. The tube was then removed, cooled in ice for 1 min. and the intensity of the colour produced read against a reagent blank at wavelength 660 mμ using the Hilger instrument. Since there was a day to day variation in the colour intensity produced by a given amount of steroid a set of standards (10 μg., 30 μg. and 50 μg. of dehydroepiandrosterone) were included with each assay.

Zimmermann reaction.

The modification of Callow, Callow and Emmens (1938) has been used.

To the steroid dissolved in 0.2 ml. ethanol 0.2 ml. of 2% m-dinitrobenzene in absolute alcohol and 0.2 ml. of 2.5 N-potassium hydroxide in absolute alcohol were added. After incubation in the dark in a water bath maintained at 25°C. for 60 min. 4 ml. of ethanol were added and the solution read against a reagent blank using 1 cm. cells at 10 mμ intervals between the wavelengths 410 mμ to 560 mμ.

Spectra/

Spectra of sulphuric acid solutions of steroids.

The procedure described by Zaffaroni (1950) was followed.

To between 70 μg. and 100 μg. of the steroid in a glass-stoppered test tube 4 ml. of concentrated sulphuric acid were added, the tube stoppered and placed in a water bath maintained at 25°C. for 2 hr. The resulting solution was then read against a sulphuric acid blank between the wavelengths 220 to 660 mμ using 1 cm. cells in a Unicam S.P.500 spectrophotometer.

(i) Acetylation of steroids.

All acetylations have been performed at room temperature. Two procedures have been used. For micro amounts of steroids (less than 100 μg.) the method of Zaffaroni and Burton (1951) has been employed. To the steroid contained in a small glass-stoppered test tube 5 drops each of acetic anhydride and pyridine were added and the mixture left overnight. The solvents were removed <u>in vacuo</u> with the aid of methanol and the acetylated steroid dried <u>in vacuo</u> over conc. sulphuric acid.

For the acetylation of larger amounts of steroids/

steroids the substance was transferred to a 25 ml. glass stoppered flask and the required amount of pyridine and acetic anhydride added. After standing overnight the flask was filled with ice-cold water and the solution filtered. The acetylated steroid was well washed with water and dried *in vacuo*.

(j) Melting point determinations.

All melting points, unless stated otherwise, were determined on a hot-stage microscope apparatus of the type described by Klyne and Rankeillor (1947) and are uncorrected.

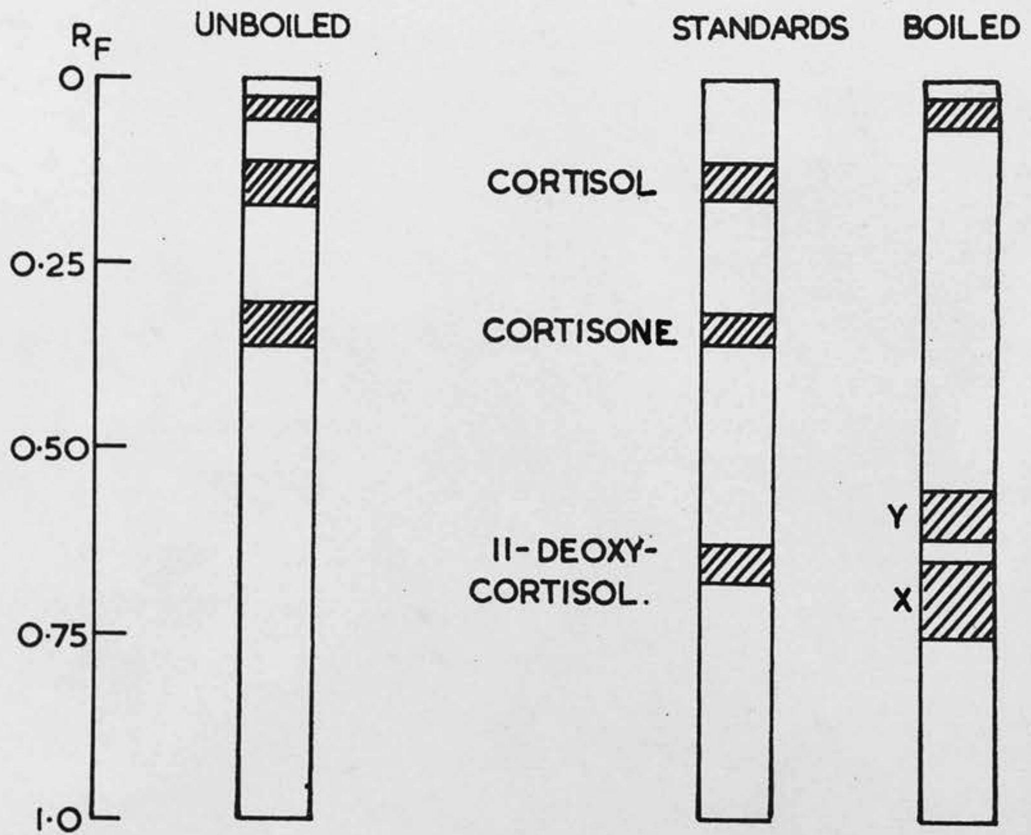


FIG.5. PAPERGRAMS OF EXTRACTS OF UNBOILED AND BOILED URINE SPRAYED WITH BLUE TETRAZOLIUM. SYSTEM. B5M.

3. Quantitative estimation of BT reducing steroids in extracts of unboiled and boiled urine.

Fig. 5 shows a typical chromatogram of extracts of unboiled and boiled urine sprayed with BT after development in system B5M. The positions of reference steroids are also shown. Cortisone, cortisol and a more polar compound (most probably 3α:17α:21-trihydroxy-5β-pregnane-11:20-dione) were regularly present in extracts of unboiled urine. Three spots were also regularly present in extracts of boiled urine; compounds X and Y of a polarity similar to 11-deoxycortisol and a more polar compound.

A rough indication of the amount of a substance present on a paper chromatogram can be obtained from the size of the spot and its intensity after the application of a suitable colour reaction. This method is only approximate and is especially inaccurate when BT is used since Chen, Wheeler and Tewell (1953) have shown that either one or two molecules of an α-ketolic steroid may react with BT. When completely reduced BT gives rise to a dark blue formazan but when only one half of the BT molecule is reduced/

reduced the formazan produced is red. Thus when BT is used to estimate steroids quantitatively it must be present in large excess so that only the semi-reduced red formazan is formed. Cope and Hurlock (1954) found that the formazan produced from BT was readily soluble in acid pyridine and could be eluted from paper chromatograms with this solvent. The intensity of the colour at the wavelength of maximum absorption was then measured. Their recoveries ranged from 75% to 160% which is not very satisfactory. This method also proved unsatisfactory when tried by the author. Known amounts of cortisone were applied to a piece of chromatography paper, sprayed with BT, allowed to dry and equal areas of paper containing the spots cut out. A sprayed piece of paper of equal area was used as a blank. The pieces of paper were placed in a glass-stoppered tube with 5 ml. of acid-pyridine (1 vol. conc. hydrochloric acid to 9 vols. pyridine) and left overnight. The next morning the solutions were filtered and the colour intensity measured at wavelength 520 mμ. As can be seen from Fig. 6 the curve relating optical density of the coloured solution to the amount of steroid used was not linear suggesting that when a large amount of steroid was used some of it did not/

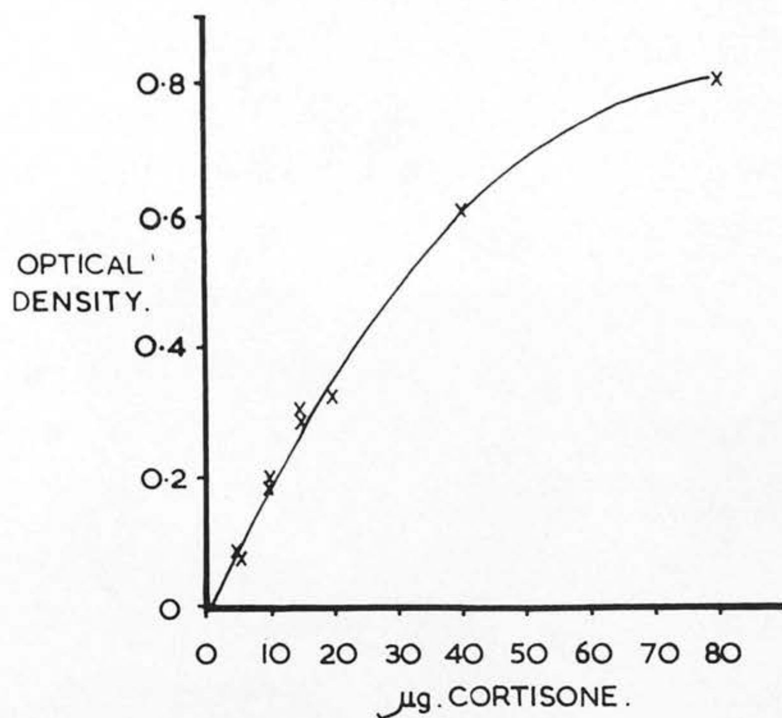


FIG.6. STANDARD CURVE OBTAINED USING THE METHOD OF COPE AND HURLOCK.

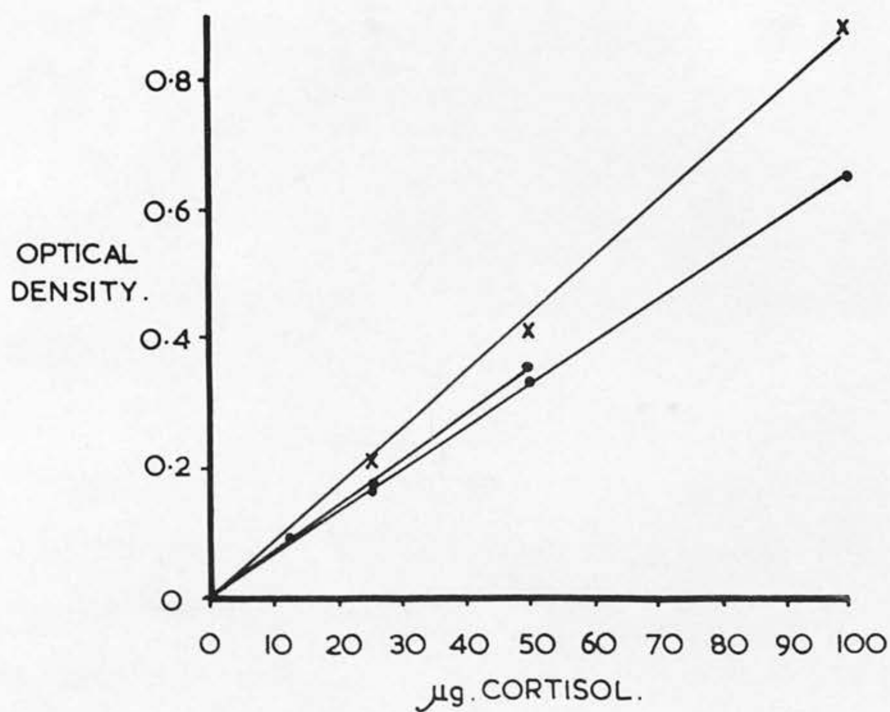


FIG.7. STANDARD CURVES OBTAINED USING THE METHOD DESCRIBED IN THIS THESIS.

not react with BT.

It was thought that a better method of estimating the steroids after chromatography would be to elute the steroids before spraying and then to carry out the BT reaction of Chen, Wheeler and Tewell (1953). Varying amounts of cortisol were applied to chromatography paper and the positions of the spots located using an ultraviolet light. Equal areas of paper containing the spots of cortisol were cut out and placed in test tubes containing 5 ml. of methanol. After standing overnight the methanol was filtered into a clean tube and the first tubes rinsed with methanol which was also filtered. The BT reaction was carried out on the residues obtained after removal of the methanol *in vacuo*. Fig. 7 shows the results obtained. Although for any one set of determinations Beer's Law was followed over a wide range of concentration day to day variations occurred so that it was necessary to run a standard compound with each set of determinations.

For the determination of compounds X and Y, cortisone and cortisol in normal male urine 7/12 of a 24 hr. specimen were extracted, boiled and re-extracted as described in Section 2. One seventh/

seventh of each residue, obtained after the partition procedure, from the 'unboiled' and 'boiled' extracts was used for the determination of formaldehydogenic substances (FSS) by the procedure described in Part 2. Aliquots each of 3/7 of the residue were applied to chromatography paper accompanied by two 30 µg. portions of cortisol as shown in Fig. 8. After development in system B5M the solvent front was marked and the paper cut lengthways in half. One half was sprayed with BT and the positions of the spots which appeared were marked on the unsprayed half of the chromatogram. Equal areas of paper containing the compounds were cut out and treated as described above. A piece of paper of equal area containing no extract was cut out and used as a blank. The results are shown in Table 6.

Table 6. /

Table 6. Quantitative determination of BT reducing compounds and FSS in extracts of unboiled and boiled urine.

		Urine 1	Urine 2	Urine 3
Cortisone	µg. per 24 hr.	10.5	38.5	30.5
Cortisol		15.5	43.0	-
Compound Y		20.5	51.5	25.0
Compound X		156.0	158.0	76.0
'Unboiled' FSS	µg. steroid per 24 hr.x	137	396	473
'Boiled' FSS		316	327	253

x A conversion factor of 11.4 (calculated for a C-21 steroid of molecular weight 340) has been employed to convert µg. of formaldehyde to µg. of steroid.

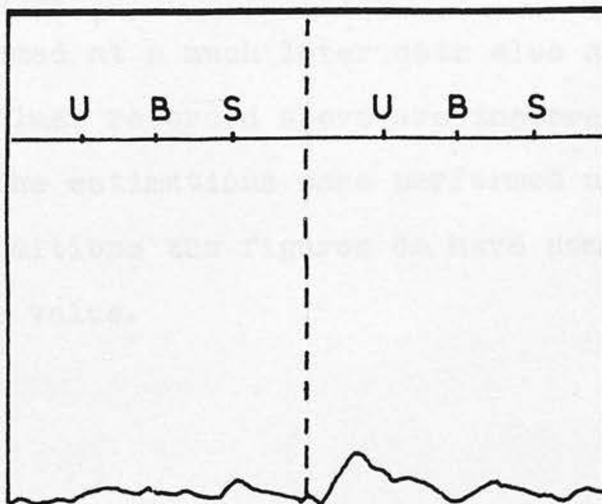


Fig. 8. Application of urine extracts to paper chromatograms.
U Extract of unboiled urine
B Extract of boiled urine
S Reference steroid

The opportunity arose of studying the effect of ACTH on the excretion of Compounds X and Y. The subjects were two males with coronary disease. The days on which ACTH was injected and the results are shown in Table 7. The increased excretion of Compounds X and Y in these experiments supported the view that X and Y were steroidal and related to the adrenal cortex.

It should be pointed out here that the above values for the excretion of X and Y do not represent the total amount of these compounds present in the urine. In all the above experiments the urine was boiled for only half an hour. As shown in Section 5 this would not be long enough to hydrolyse completely the conjugates of X and Y. Work performed at a much later date also shows that the values recorded above are incorrect but since all the estimations were performed under similar conditions the figures do have some comparative value.

Table 7. /

Table 7. Quantitative determination of BT reducing compounds and FSS in extracts of unboiled and boiled urine from males injected with ACTH.

Male 1

		Day 1	Day 2	Day 3
Cortisol		52	640	66
Cortisone	μg. per 24 hr.	26	137	60
Compound Y		56	155	78
Compound X		150	467	120
'Unboiled FSS'	μg. steroid^{x} per 24 hr.	570	1228	338
'Boiled FSS'		275	1424	506

100 i.u. of ACTH injected intramuscularly on the morning of Day 2.

Male 2

		Day 1	Day 2	Day 3	Day 4	Day 7
Cortisol		32	600	672	1900	13
Cortisone	μg.	16	80	16	160	33
Compound Y	per 24 hr.	25	88	144	132	26
Compound X		100	400	336	320	70
'Unboiled FSS'	μg. ster- oid	378	1183	550	1512	125
'Boiled FSS'	per 24^{x} hr.	400	252	288	1100	80

100 i.u. of ACTH injected intramuscularly on the morning of Day 2, Day 3 and Day 4.

x A conversion factor of 11.4 (calculated for a C-21 steroid of molecular weight 340) has been employed to convert μg. of formaldehyde to μg. of steroid.

Although no recovery experiments have been performed it is thought that the method described above is fairly accurate. The optical density of the solution resulting from the reaction of BT and the cortisol standard used in the above experiments agrees well with the optical density of the solution obtained by the reaction of BT with cortisol which has not been chromatographed and eluted. Zaffaroni (1953) has shown that elution of steroids from paper by methanol is quantitative. The extraction of urine with 2 vols. of $CHCl_3$ should remove all steroids as polar as cortisone or cortisol quantitatively; it may be necessary however to use ethyl acetate to extract the very polar highly oxygenated steroids. It has been shown by Dr A. Colàs that no losses occur during the partition procedure. In addition the values found for the excretion of cortisol and cortisone agree well with the values reported by Burton, Zaffaroni and Keutmann (1951), de Courcy, Bush, Gray and Lunnon (1953) and Cope and Hurlock (1954).

4. The stability of X and Y to acid.

The stability of the compounds to both hot and cold acid was determined. A sample of X obtained/

obtained by paper chromatography of extracts of boiled urine was dissolved in 350 ml. of 1% methanol in water and the solution divided into seven equal parts. One part was unacidified, a second was boiled for 20 min. with 15 vols.% conc. hydrochloric acid and the remainder acidified to pH 1 with sulphuric acid and allowed to stand at room temperature for 0, 2, 4, 7 and 24 hr. After the treatment described above each solution was extracted and prepared for chromatography. The results showed that after hot acid treatment very little BT reacting material remained and part of the material remaining ran with the solvent front on chromatography. Standing for 2 hr. or more at pH 1 transformed X into a compound with the same polarity as Y and this tranformation product, called Xa, was stable to cold acid.

A similar experiment performed with Y showed that it too was destroyed by hot acid but was stable to cold acid.

The destruction of X by hot acid was not surprising in view of the fact that X reduced BT and therefore was assumed to have an α-ketol group. Since the transformation caused by cold acid did not result in a decreased power to reduce BT some part/

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part of the molecule of X other than the α-ketol group must be involved. The effect of acid could be explained if it was assumed that X was a steroid with the 3:5-cyclo-6β-hydroxy structure. (Other evidence supported this assumption). It was shown by Dingemanse, Huis in't Veld and Hartogh-Katz (1948a,b) that i-androstanolone (3:5-cycloandrostan-6β-ol-17-one) could be isolated from urine when the urinary conjugates were hydrolysed by boiling at neutral pH and the steroids liberated continuously extracted with benzene. Treatment with 5% hydrochloric acid at room temperature produced 80% of 3β-chloro-androst-5-en-17-one and 20% of dehydro*epi*-androsterone (Dingemanse, Huis in't Veld and Hartogh-Katz, 1948b). The chloro-compound is less polar than the cyclo-compound so that if X was a cyclo-steroid the cold acid transformation product could not be the chloro-derivative but would have to be the steroid with the 3β-hydroxy-5-ene structure. As noted above i-androstanolone produces less than 20% of dehydro*epi*androsterone under these conditions.

These transformations are shown in Fig. 9 and are taken from the paper by Teich, Rogers, Lieberman/

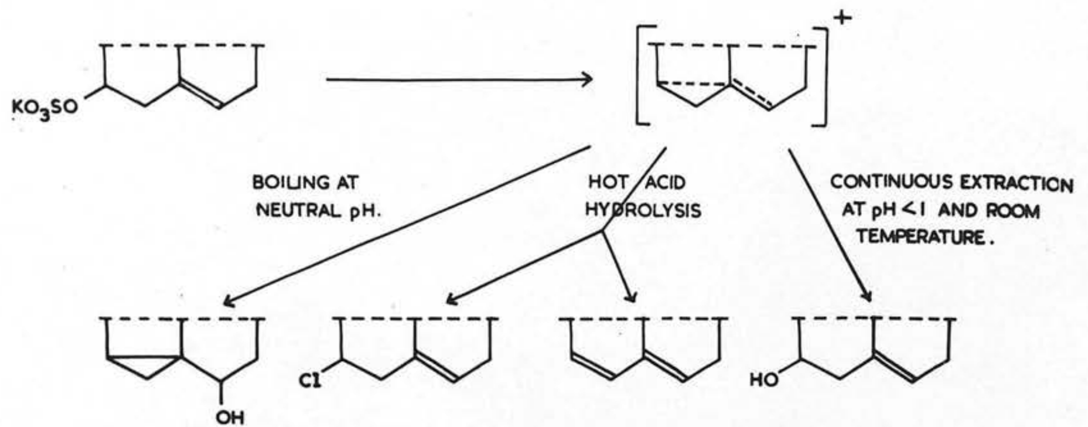

FIG.9. HYDROLYSIS OF DEHYDROEPIANDROSTERONE SULPHATE.

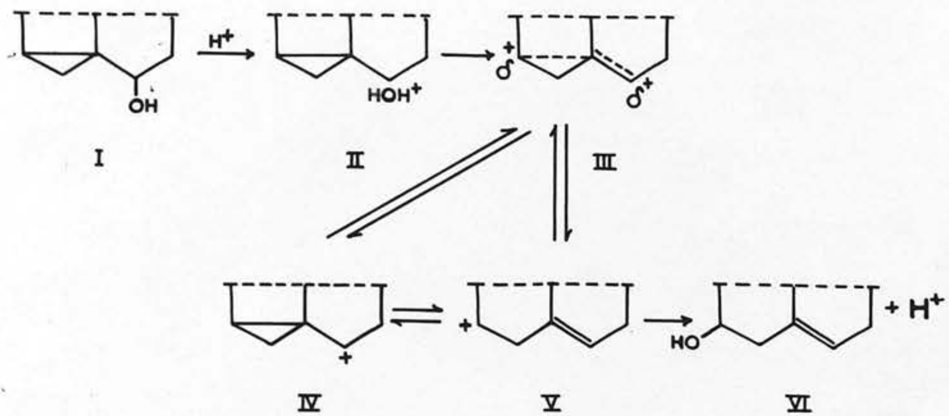

FIG.10. EFFECT OF ACID ON A 3:5-CYCLOSTEROID.

Lieberman, Engel and Davis (1953). These authors have suggested that the steroid sulphate which occurs in urine is dissociated by heat with the formation of a carbonium ion which has a resonating structure. The product or mixture of products obtained from the hydrolysis depends upon the conditions of hydrolysis.

In the above experiments on the lability of X to acid at room temperature sulphuric acid had been used. The suggestion that X may be a cyclo-steroid was strengthened by its behaviour during the Girard separation. In the work of Dingemanse _et al_. quoted above, during the Girard separation 80% of i-androstanolone was transformed to the chloro-derivative and 20% to dehydro_epi_androsterone. When the Girard separation was performed with Compound X 30%-50% was transformed to Xa and the remainder was transformed to a BT reducing compound which runs with the solvent front during paper chromatography. This non-polar derivative could well be the chloro-compound. Because of the above transformations sulphuric acid has been used to hydrolyse the Girard hydrazones in the present work. Using sulphuric acid the product appears to/

to be entirely Xa. Wolff and Wallis (1952) have suggested the scheme shown in Fig. 10 for the conversion of the cyclo-steroid by acid. The hydroxyl group of the cyclo-steroid (I) is protonated with the formation of II. The hydroxyl group is thus removed with the formation of the carbonium ion III. The limiting structure V is favoured is an acid medium and this reacts with water to form the hydroxy-steroid VI. When hydrochloric acid is used V must react more quickly with chloride ions than with hydroxyl ones producing predominantly the chloro-steroid. It has been shown that ethers of the cyclo-steroid react with halogen acids in acetic acid solution at room temperature forming the halide of the normal steroid (Beynon, Heilbron and Spring, 1936) but with sulphuric acid in acetic acid the acetate of the normal steroid was formed (Wagner, Wolff and Wallis, 1952).

As reported above chromatograms of boiled urine showed a BT reducing spot Y which is slightly more polar than X and the relative amounts of X and Y present were approximately the same for different urines. Dingemanse (1952) found

that/

that i-androstanolone obtained by hydrolysis of the urinary conjugate was always accompanied by about 10% of dehydroepiandrosterone depending upon the pH. Thus it could be that Y and Xa are identical but there is no conclusive evidence for this assumption.

5. Liberation of formaldehydogenic substances (FSS) by boiling.

It was known that when pre-extracted urine was boiled for periods of time varying from 10 to 40 minutes the amounts of FSS extractable varied only slightly. It was possible however that reboiling the urine after extraction would liberate further amounts of FSS. To test this possibility a 24 hr. sample of urine was divided into two equal parts A and B after being extracted with chloroform and treated as shown below:-

A.	B.
Divided into 4 equal parts	
A1 boiled ¼ hr.	B1 boiled ¼ hr. extracted with $CHCl_3$
A2 boiled ½ hr.	B2 extracted (B1) urine boiled ½hr., extracted with $CHCl_3$
A3 boiled 1½ hr.	B3 urine from B2 boiled ½ hr., extracted with $CHCl_3$

A4/

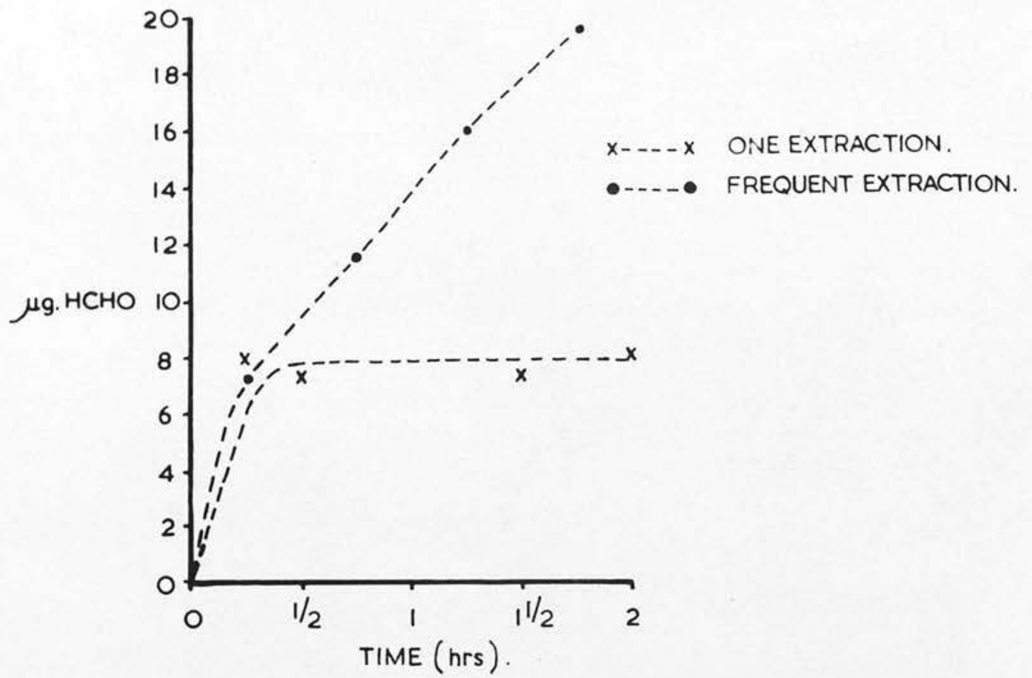

FIG.11. LIBERATION OF FSS BY BOILING.

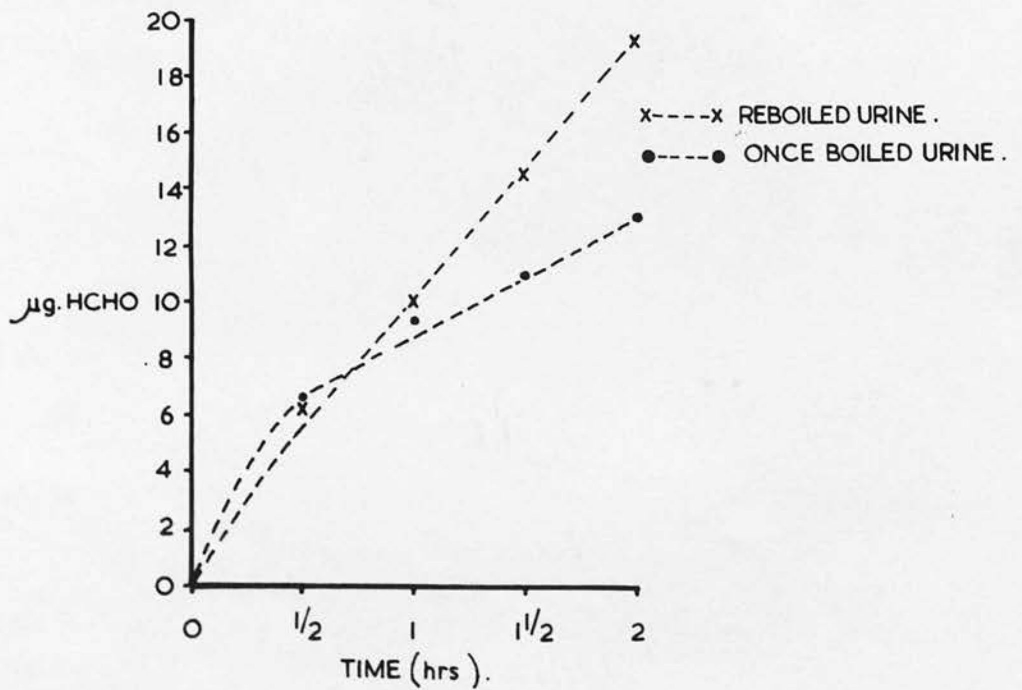

FIG.12. LIBERATION OF FSS BY BOILING.

A4 boiled 2 hr.	B4 urine from B3 boiled $\frac{1}{2}$ hr., extracted with $CHCl_3$
Each part extracted with $CHCl_3$ for determination of FSS	$\frac{1}{4}$ of each $CHCl_3$ extract used for determination of FSS

The results are shown in Fig. 11, frequent chloroform extraction appeared to favour the release of larger amounts of FSS. A similar result was obtained when the experiment was repeated in a slightly different manner. One half of a sample of urine was boiled for four periods of $\frac{1}{2}$ hr. and extracted at the end of each period of boiling (extracts A1, A2, A3, A4). The other half of the sample was boiled for $\frac{1}{2}$ hr., extracted and this extracted urine allowed to stand at room temperature and extracted at half hourly intervals (B1, B2, B3, B4). The values for FSS obtained are shown in Fig. 12. Thus boiling for a short period of time does not cause complete hydrolysis of the conjugated FSS.

Paper chromatography showed that reboiling of urine also liberated more of Compounds X and Y. When a sample of urine was boiled for three periods of one hour and extracted at the end of each period, even the third extract contained large/

large amounts of X and Y. Some spots which fluoresced in ultraviolet light but did not reduce BT were also present. Some of these appeared in the second and third extracts but not in the first. When urine was refluxed for three periods of 1 hr. with toluene, fresh toluene being used for each period, the third toluene extract still contained Compounds X and Y.

As already noted Dingemanse *et al*. have isolated i-androstanolone by continuous extraction of hot neutral urine with benzene. Using 1/3 vol. of benzene two or three periods of heating for five hours were needed for complete hydrolysis and extraction of the steroid. Jensen and Totterman (1952) have described a two stage hydrolytic procedure for 17-ketosteroid conjugates in urine. The first stage consisted of heating the urine at 100°C. without continuous extraction. It was shown that after heating for 6 hr. the yield of the dehydro*epi*androsterone fraction remained practically constant and that no further amounts of dehydro*epi*-androsterone were released by subsequent acid hydrolysis. Changes in pH of the urine from 3 to 11 did not affect the amount of dehydro*epi*androsterone detected in the first stage. Talbot, Ryan and/

and Wolfe (1943) and Bitman and Cohen (1951) have also described methods of hydrolysing urinary 17-ketosteroid conjugates by heating at pH 5.8 and 4.7 respectively. Talbot _et al_. using an aqueous solution of sodium dehydro_epi_androsterone sulphate showed that hydrolysis was complete after four hours' heating at 100°C. and the recovery of 17-ketosteroid was satisfactory at any pH between 4 and 7. Bitman and Cohen found that hydrolysis was only 85% complete in four hours and that twelve hours were needed for complete hydrolysis. The pH at which the urine was heated had little effect until a pH of 3 or less was reached; the quantity of 17-ketosteroids liberated between pH 4-6 was approximately constant and accounted for about 25% of the 17-ketosteroids liberated by acid hydrolysis. Using synthetic steroid sulphates only those containing the sulphate group at the 3β-position and unsaturated at position 5 were hydrolysed. About one half of the 17-ketosteroids in a urine hydrolysate were precipitated by digitonin and dehydro_epi_androsterone was isolated from this fraction. 3-Chloro-androst-5-en-17-one was isolated from the fraction not precipitated by digitonin.

Thus/

Thus the values quoted in Section 3 for the excretion of Compounds X and Y must be minimal ones. It would appear that a continuous extraction of hot urine by benzene for at least 5 hr. would be necessary for complete hydrolysis and extraction of X and Y.

6. Acetylation of X.

Zaffaroni and Burton (1951) have shown that it is possible to decide whether a compound has one or more than one hydroxyl group that can be acetylated by the difference in R_F value of the free and acetylated compound. If a steroid has only one hydroxyl group that can be acetylated the R_F value of the acetylated compound is only slightly greater than that of the free compound. If more than one hydroxyl group can be acetylated the resulting acetate is usually a non-polar compound and runs near the solvent front.

About 100 μg. of X was obtained by eluting papergrams of boiled urine extracts. 15 μg. were again chromatographed on paper to check its purity and the remainder acetylated. The R_F values obtained for X, 11-deoxycortisol and 11-dehydrocorticosterone and their acetates in various chromatographic/

chromatographic systems are shown in Table 8. From these values it is clear that X must contain at least two hydroxyl groups.

Table 8. R_F values for compound X, reference steroids and their acetates in various chromatographic systems.

Substance	System		
	B2	B1	A
X	0.37		
X acetate		1.0	0.86
11-deoxycortisol	0.32		
" acetate		0.70	-
11-dehydrocorticosterone	0.49		
" acetate		0.75	0.04

7. Digitonin precipitation of X and Xa.

One half of a sample of X (about 200 μg.) was treated with acid to form Xa. Both X and Xa were then subjected to the digitonide formation procedure. Xa appeared in the β-fraction while X appeared in the α-fraction.

Thus bearing in mind the limitations of the method described in Section 2 it was probable that Xa contained a 3β-hydroxyl group which was absent in X. X and Xa behaved in this test in a similar/

similar manner to i-androstanolone and dehydro-*epi*androsterone.

The probability that Xa contained a 3β-hydroxyl group was strengthened by comparing the reaction of Xa and dehydro*epi*androsterone in the Pettenkofer reaction; approximately equal amounts of the two compounds produced the same intensity of colour.

8. Summary.

It has been shown that chloroform extracts of boiled urine contain substances which reduce BT and which do not occur in chloroform extracts of unboiled urine. Compounds X and Y have been found in all urines examined and are the BT reducing substances present in largest amounts. A very polar compound was also usually present and occasionally other substances in small amounts. After the administration of ACTH not only was the excretion of X and Y increased but many other BT reducing substances were detected on chromatograms. Since neither X nor Y absorbed ultraviolet light of wavelength 254 mμ or gave a positive sodium hydroxide fluorescence reaction (Bush, 1952) they were unlikely to contain an αβ-unsaturated ketone group. If X and Y were steroids the positive BT reaction/

reaction suggested they were C-21 steroids with an α-ketol side-chain and this suggestion appeared likely since extracts containing X and Y liberated formaldehyde on periodate oxidation. From their chromatographic behaviour it was likely that they contained four oxygen atoms as do corticosterone, 11-dehydrocorticosterone and 11-deoxycortisol which have similar R_F values to X and Y. One of these oxygen atoms must be present as a ketone group for X and Y are found in the ketonic fraction on performing the Girard separation. Acetylation suggested that at least two hydroxyl groups were present, one presumably at C_{21} and from the digitonin precipitation and Pettenkofer reaction of Xa the other was present at position 3. The mode of hydrolysis suggested that X and Y arose from a conjugate esterified at position 3 and with a double bond at position 5. If so the transformation by acid was explained as conversion of the cyclo-steroid produced by the hydrolysis to the normal compound. No 17-ketosteroid was detected after sodium bismuthate oxidation suggesting that a hydroxyl group was not present at position 17.

By working up about 400 litres of normal male/

male urine Colàs (1955) isolated two crystalline compounds. The first, K1, was shown to be 3β-chloro-androst-5-en-17-one and 4.5 mg. of the second, designated K2, were obtained. The method of isolation consisted of a Girard separation of the residue from the partition procedure and chromatography of the ketonic fraction on a Celite-magnesium silicate column. Those fractions shown by paper chromatography to contain Xa were combined and rechromatographed on a small Celite-magnesium silicate column. K2 had a melting point of 177-181°C., was not formaldehydogenic, gave a negative Zimmermann reaction, and under the conditions of the reaction then employed did not appear to reduce BT. With concentrated sulphuric acid an intense purple colour with a green fluorescence was produced and the absorption spectrum showed maxima at 271, 378, 400 and 560 mμ , minima at 250, 315, 390 and 458 mμ and an inflection at 496 mμ . Little could be deduced from the spectrum except that K2 probably contained a 5-en-3β-ol group. A quantitative carbon and hydrogen analysis left the empirical formula in doubt. The infrared spectrum suggested that a progesterone-like side-chain or ketone groups in positions 3 or 17 were absent. A strong band at 1740 $cm.^{-1}$

suggested/

suggested a cyclo-pentanone and since K2 gave a negative Zimmermann reaction it was assumed that the ketone group was present at position 16. The mother liquors from which K2 had been crystallized and which were expected to contain X did not appear to reduce BT.

The residue from a second pool of urine was fractionated in a slightly different manner. The residue was first chromatographed on a magnesium silicate-Celite column and those fractions shown to contain Compounds X and Y were combined and subjected to a Girard separation. The ketonic fraction was then chromatographed on a small column of magnesium silicate-Celite and the fractions containing Xa combined. Three crystallizations from methanol gave 7.9 mg. of K2. K2 was shown to give a positive Pettenkofer reaction and to be precipitated by digitonin. Again it did not appear to reduce BT but the mother liquors from which K2 had been crystallized did. The analysis of K2 was again not helpful. The nearest possible empirical formula to fit the analysis was $C_{21}H_{30}O_4$ but the agreement was not very good.

Thus although some of the properties of X and Xa were known, their isolation was apparently not/

not achieved. K2 was assumed to be a steroid with a 5-en-3β-ol grouping and with a ketone group at position 16. The number of carbon atoms present in its molecule was unknown.

9. Fractionation of the first pool of urine.

The residue obtained from 165 litres of urine weighed 1.06 g. After chromatography on magnesium silicate-Celite the X fraction weighed 0.756 g. This was subjected to a Girard separation and the hydrazones formed hydrolysed with hydrochloric acid. The weights of the fractions were:-

Non-ketonic fraction	308.7 mg.
Ketonic fraction	222.0 mg.

The ketonic fraction was rechromatographed on magnesium silicate-Celite. Paper chromatography of the fractions from the column showed that during the Girard separation about half of the Compound X had been converted to Xa and the remainder to a non-polar compound running with the solvent front, presumably the 3-chloro-derivative of X. The fractions containing these two compounds were combined since complete separation had not been obtained and rechromatographed on a small column of magnesium silicate-Celite. The two/

two compounds were completely separated and the weight of the combined Xa fractions was 67.5 mg. whilst that of the non-polar fractions was 87.1 mg.

(a) Examination of the non-polar material.

The isolation of K3.

The residue containing the non-polar material was dissolved in 0.5 ml. of methanol and left at -20°C. to crystallize. The crystals which formed were removed by filtration and designated K3. After dissolving in methanol, filtering and re-crystallizing twice more the white crystals weighed 11.7 mg. The melting point was recorded:-

154°-160°	Softening of the crystals occurring at the edges.
167°	Slowly beginning to melt.
170°	Melting becoming more rapid.
176°	Melt complete.

A sample was dried to constant weight for analysis and gave the following figures:-

C, 72.58% H, 8.26% Cl, 11.5%

There was no residue after combustion. The result of the chlorine analysis may be 0.4% high since the analysis of known compounds gave values for chlorine in error by this amount.

Thus possible formulae for K3 are:

$C_{21}H_{29}Cl$	C, 72.3%	H, 8.37%	Cl, 10.17%
$C_{19}H_{27}O_2Cl$	C, 70.7%	H, 8.37%	Cl, 11.0%

Due/

Due to the small amount of material available K3 was not investigated further. Additional comments regarding the structure of K3 appear in later sections of this thesis.

When 1/150 of the residue obtained from the mother liquors of the first crystallization of K3 was chromatographed in system B2 a large BT reducing zone, representing about 50 µg. (visual estimation), was seen at the solvent front.

The isolation of K4.

Since the isolation of K3 appeared not to have resulted in the loss of any of the BT reducing material which had been presumed to be the chloro-derivative of Xa, an attempt was made to isolate this compound from the mother liquors of the first crystallization of K3. One half of the residue from the mother liquors (weight 42.2 mg.) was chromatographed on a 15 x 1 cm. column of Celite using methanol:water - 8:2 as the stationary phase. The mobile phase was petroleum ether (boiling range 80-100°C.). Eleven fractions of 5 ml. each were collected and 1/50 of each fraction chromatographed on paper in system A. The only BT reducing spots detected were in fractions 2, 3 and 4 and all had an R_F value of 0.70. The residue obtained from combining these/

these fractions weighed 13.1 mg. Two crystallizations from methanol gave 2.7 mg. of a slightly yellow amorphous residue with a few white plates. The melting point of the white crystals was 157-176°C. When 50 μg. of the compound were chromatographed in system A a faint BT reducing spot, representing about 2-5 μg. of an α-ketol, of R_F 0.70 was seen. Thus it was assumed that K4 was not the chloro-derivative of Xa but may have contained 5-10% of this substance as a contaminant. It seems possible that K4 is identical with K3.

The isolation of K5.

Evaporation of the solvents from fractions 8 and 9 of the partition column described above left a residue of crystalline material. The two fractions were combined (weight 5.0 mg.) and crystallized from 0.05 ml. of methanol to give 4.6 mg. of white needles. The melting point was recorded:-

137° Beginning to melt.

139° Resolidifies.

144° Beginning to melt again.

147° Melt complete.

The/

The melting point behaviour was similar to that of dehydroepiandrosterone; Butenandt, Dannenbaum, Hanisch and Kudszus (1935) record the melting point as 137-138°, 148°; Hirschmann and Hirschmann (1947) 139.5, 147.5-149°C. and Lieberman, Dobriner, Hill, Fieser and Rhoads (1948) give 137-138, 145-146°C. There was no depression of the melting point when authentic dehydroepiandrosterone was mixed with K5. It was thus concluded that K5 was dehydroepiandrosterone.

(b) Examination of the Xa fraction.

The isolation of K6.

Colàs had isolated only small amounts of K2. This was in part due to the number of crystallizations that were necessary to produce a pure product. It seemed possible that the number of crystallizations could be reduced if a purer product was obtained from column chromatography. A partition column seemed most suitable for purifying the Xa fraction.

Cox (1952a) had found that the partition coefficient for 11-deoxycortisol in the solvent system methanol, water, benzene, 8:2:10 to be 5.3. Since Xa was slightly more polar than 11-deoxycortisol this system should be suitable for/

for the purification of Xa. The residue (weight 67.5 mg.) of the Xa fraction was accordingly chromatographed on a 32 x 2 cm. column of Celite using the above solvent system. Fractions 9 and 10 of the eluate after removal of the solvent were crystalline. Those fractions (numbers 9-15) shown by paper chromatography to contain Xa were combined. The weight of the residue obtained was 30 mg. This was crystallized three times from methanol and once from benzene producing 7.5 mg. of crystals which had the same form as K2. The melting point was determined:-

90-95°	Went opaque
172°	Beginning to melt slowly
175°	Melting becoming more rapid
181°	Melt complete

This was similar to the melting point of K2 and the melting point of a mixture of K2 and K6 showed no change from that recorded for K6. Thus it was concluded that K6 and K2 were identical. This was confirmed by the spectra of the sulphuric acid chromogens. Both compounds when dissolved in conc. sulphuric acid produced a pink solution with a green fluorescence which had the/

the following features:-

	K2	K6
Maxima	271,378,400,560	270,380,490
Minima	250,315,390,458	250,300,460
Inflections	496	400,490

An aliquot of the mother liquors from the crystallization of K6 and 20 µg. of crystalline K6 were chromatographed on paper. The crystalline K6 showed a BT reducing spot of about 3 µg. of the polarity of Xa while the mother liquors showed a large zone of BT reducing material (about 50 µg.) of the same polarity. Thus it seemed to appear that K6 might still contain Xa as an impurity and this would account for the wide range of temperature over which K6 melted.

Chromatography of the acetate of Xa.

Katzenellenbogen, Dobriner and Kritchevsky (1954) have described a system suitable for the chromatography of non-polar compounds on a column. The supporting material is silica gel with ethanol (4 ml. per 10 g. of silica gel) as stationary phase and petroleum ether, methylene chloride 1:1 with the addition of 1% ethanol as mobile phase.

The/

The residue (wt. 4.8 mg.) from the mother liquors of the K6 crystallization was acetylated at room temperature overnight. On chromatography on silica gel, BT reacting material was eluted between 10 and 22 ml. (A1) and by washing the column with ethanol (A2). Total amount of A1 obtained was 2 mg. and the amount of A2 was smaller than this. Both fractions were twice recrystallized from methanol but only waxy residues were obtained. When the BT reaction was performed on paper with 1/10 of each residue A1 gave a strong reaction and A2 a weak one.

Due to the small amounts of material available it is not possible to make any conclusion regarding the nature of A1 and A2. It is possible that A1 was the diacetate of Xa whilst A2 could have been the monoacetate.

10. Fractionation of the second and third pools of urine.

From the second pool consisting of 187 litres of urine 50 mg. of crude material were obtained after chromatography. Three crystallizations from benzene gave 28.8 mg. of crystalline K2 shown by the melting point to be slightly less pure than the previous batch and the residue from the/

the combined mother liquors weighed 22.4 mg.

Two 25 μg. aliquots of each fraction were chromatographed on paper in system B2 with 11-deoxycortisol as a reference compound. One half of the paper was sprayed with BT and the other half with the dinitrophenylhydrazine reagent described by Kochakian and Stidworthy (1952). It was considered that the paper sprayed with BT would reveal only Compound Xa in the mother liquor residue and the 11-deoxycortisol standard since K2 at that time was thought not to be BT reducing. K2 being ketonic should react with dinitrophenylhydrazine so that the mother liquor residue should show two spots Xa and K2 when sprayed with this reagent. Spraying with BT showed that about 7 μg. of Xa were present in the crystalline K2 and about 4 μg. in the mother liquor residue. On spraying with the dinitrophenylhydrazine 11-deoxycortisol gave an orange spot characteristic of an αβ-unsaturated ketone and only two yellow spots, characteristic of an isolated ketone group, were observed and these corresponded with the BT reducing spots.

Thus the expected result was not obtained. The results could be explained by assuming that Xa/

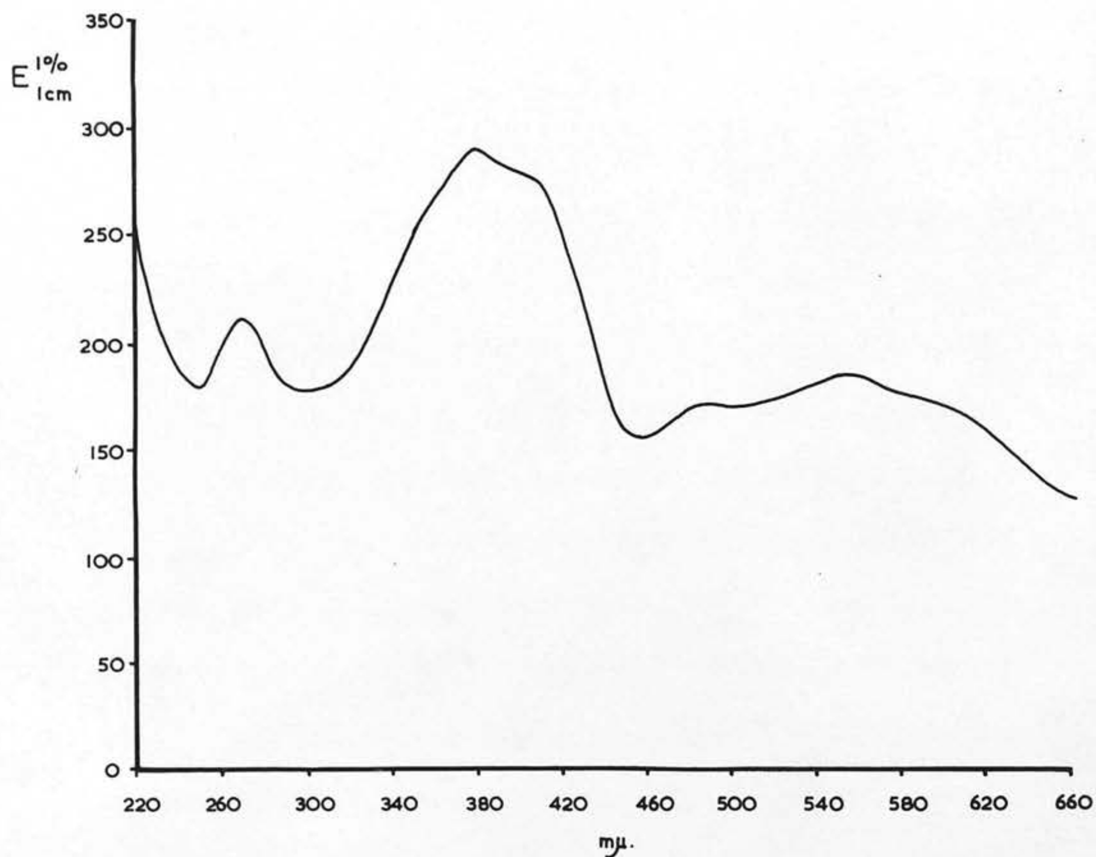

FIG. 13. ABSORPTION SPECTRUM OF K_2 IN CONC. H_2SO_4.

Xa and K2 had exactly identical chromatographic properties or that the ketone group in K2 was for some reason unreactive to dinitrophenylhydrazine.

From the third pool of urine (201 litres) 16 mg. of pure K2 were obtained and the mother liquor residue weighed 64.8 mg. The K2 from the 2nd and 3rd pools was combined with that remaining from the 1st pool and with 7.9 mg. donated by Dr Colàs. The total weight of K2 so obtained was 38.6 mg. This was crystallized once from 50% aqueous methanol and once from benzene to give 27.2 mg. of pure K2 (melting point 176-180°C.) and a residue of 8.1 mg. from the mother liquors.

Properties of K2.

The spectrum of the sulphuric acid chromogen of K2 is recorded in Fig. 13; it did not differ from that recorded by Colàs for his sample of K2.

When 56 µg. of K2 were chromatographed in system B2 with 20 µg. of 11-deoxycortisol as reference steroid spraying with BT showed a blue spot corresponding to approximately 5 µg. of an α-ketol and with an R_F value similar to that of Xa. Thus it was assumed that K2 contained about 10% /

10% of Xa in spite of its extensive purification. The oxidation of 112 μg. of K2 with periodic acid did not produce any acetaldehyde (Cox, 1952b) but did produce 1.4 μg. of formaldehyde. Assuming a conversion factor of 11.4 (calculated for a C_{21} ketolic steroid of molecular weight 340) this amount of formaldehyde corresponded to 16 μg. of steroid confirming the result obtained with BT. However the formaldehyde detected in this experiment must be ascribed to experimental error for when this determination was repeated on another sample of K2 no formaldehyde was detected.

The Zimmermann reaction performed with 112 μg. of K2 did not show a peak of absorption between 400 and 530 mμ. confirming the result obtained by Colas.

The analysis of K2 (dried to constant weight) gave the following:-

Carbon	75.06%
Hydrogen	8.86%
Oxygen	16.08% (by difference)

Thus possible formulae for K2 could be:-

$C_{19}H_{26}O_3$	Carbon	75.46%	Hydrogen	8.67%
$C_{19}H_{28}O_3$	"	74.96%	"	9.27%

The optical rotation of K2 was measured using a micro-tube:-

$[\alpha]_D^{15}$ = +11.8 (2.875 mg. K2 in 0.5 ml. EtOH c = 0.575)

13.67 mg. of K2 were acetylated with acetic anhydride and pyridine overnight at room temperature. The isolated acetate weighed 17.6 mg. Thus the increase in weight on acetylation was 3.93 mg. and these values can be used to determine the number of acetylable groups in the molecule of K2. From the above figures:-

$$\% \frac{\text{Acetate}}{\text{Steroid}} = \frac{3.93 \times 100}{13.67} = 28.7$$

Assuming K2 to have a molecular weight of 304 (calculated for $C_{19}H_{28}O_3$) then for a mono-acetate:-

$$\% \frac{\text{Acetate}}{\text{Steroid}} = \frac{42 \times 100}{304} = 13.9$$

For a di-acetate:-

$$\% \frac{\text{Acetate}}{\text{Steroid}} = \frac{84 \times 100}{304} = 27.8$$

Thus K2 formed a diacetate and the result obtained earlier by the increase in R_F on acetylation was confirmed.

When 100 μg. of K2 diacetate were applied to paper and sprayed with BT there was a positive reaction corresponding to about 3 μg. of 11-deoxycortisol. An equal amount of the residue from the mother liquors of the K2 diacetate crystallization treated similarly gave a positive reaction equivalent to about 5 μg. of 11-deoxycortisol.

The/

The melting point of the diacetate after two crystallizations from methanol was 167-171°C. The analysis of a sample of the diacetate (no loss of weight on drying) gave the following figures:-

Carbon	71.4%	
Hydrogen	8.37%	
Oxygen	20.23%	by difference

For the diacetate of $C_{19}H_{26}O_3$:-

Carbon 71.5% Hydrogen 7.77%

For the diacetate of $C_{19}H_{28}O_3$:-

Carbon 71.1% Hydrogen 8.25%

Determination of the optical rotation of the diacetate gave the value $[\alpha]_D^{12} = +22.9$ (3.010 mg. in 0.5 ml. EtOH c = 0.602)

From the above results, especially the optical rotations, it was possible to derive new facts about the structure of K2. It seemed certain from the results of the analyses that if K2 is a steroid it has 19 carbon atoms and the probable formula is $C_{19}H_{28}O_3$. Assuming K2 to have a 5-en-3β-ol grouping and a ketone group, which infrared evidence suggested was in ring D, then another hydroxyl group has to be accounted for. Possible positions of hydroxyl groups, based/

based on the C_{19} steroids isolated from urine, are 3, 6, 11β, 16 and 17 (cf. General Introduction). Of these position 3 is excluded since it already contains one hydroxyl group and if K2 has a 5-en-3β-ol group, position 6 can also be excluded. The 11β position is possible but improbable; this position is a hindered one and acetylation of an 11β-hydroxyl group has only recently been accomplished and needs a strong acid catalyst (Oliveto, Gerold, Weber, Jorgensen, Rausser and Herschberg, 1953). However Steiger and Reichstein (1937) found that 3β:11β-dihydroxy-5α-androstan-17-one formed a diacetate on heating with acetic anhydride and pyridine and Kemp, Kappas, Salamon, Herling and Gallagher (1954) have shown that the same compound can be partially acetylated at the 11-hydroxyl group at room temperature. Thus the most likely position for the remaining hydroxyl group is at C_{16}, if this is not occupied by a ketone group, or at C_{17}.

From the BT reaction and the liberation of formaldehyde it would appear at first sight that K2 must be contaminated to the extent of 5-10% even though the sample of K2 used had been crystallized six times. Apparently also acetylation and crystallization of the acetate was not effective in/

in removing the impurity.

The contribution of substituents in the steroid molecule to the molecular rotation, M_D, for that compound has recently been well reviewed by Klyne (1955). The contribution of the 5-en-3β-ol group is about -245 and that of a 16-oxo group, which has the strongest influence of any substituent, about -450. Thus if K2 contained both of these groups or only a 16-oxo group it should be strongly laevo rotatory. The fact that K2 is slightly dextro rotatory eliminates completely the 16-oxo group. To counteract the laevo rotatory contribution of the 5-en-3β-ol group the remaining substituents must exert a dextro rotatory influence. Possible positions for such ketone groups are 7 in the 5α series (+220), 11 (+80) and 17 (+250). Position 7 is eliminated for with unsaturation at position 5 such a steroid would be an αβ-unsaturated ketone and therefore absorb in ultraviolet light which K2 does not. The 11 position can also be excluded since it has been shown that a ketone group in this position does not react with Girard's reagent (Mason, 1948). Position 17 is only possible if there is some neighbouring substituent to account for the negative Zimmermann reaction. If a ketone group is/

is present at position 17 then the position cannot also have a hydroxyl group and the most probable position for the hydroxyl group becomes position 16. The rotation contribution for a 16α-hydroxyl group varies from -30 to -160 whilst that of a 16β one is +20.

The values quoted above for the rotation contributions of the various groups assume that no vicinal action occurs (Barton and Cox, 1948). Considering the structure first assumed for K2, 3β:17β-dihydroxy-androst-5-en-16-one, it is rather unlikely that vicinal action would be strong enough to convert what was anticipated to be a laevo rotatory compound into a dextro rotatory one. This structure can be completely eliminated since the compound has been synthesized by a number of workers and its properties (m.p. 202-205°, $[\alpha]_D$ -195; Adams, Patel, Petrow and Stuart-Webb, 1956) do not coincide with those of K2. Thus a possible structure for K2 which accords with the experimental evidence is 3β:16-dihydroxy-androst-5-en-17-one, assuming that none of the substituents occurs at an unusual position in the molecule. Table 9 compares the molecular rotation contribution of the 16-hydroxyl and 16-acetoxyl group in K2, assuming it to have the above structure/

structure, with the value for these groups in other 17-ketosteroids. The results are not in disagreement with the postulated structure for K2. Only one value for a 16β-acetoxy group and none for a 16β-hydroxyl group can be calculated. On rotational evidence it is not possible to decide whether the hydroxyl group at position 16 is in the α or β configuration. Cooley, Ellis, Hartley and Petrov (1955) have synthesized the diacetates of the two possible compounds and the physical properties of these compounds are compared with those of K2 diacetate in Table 10.

Table 9. /

<u>Table 9.</u> Molecular rotation contribution of hydroxyl groups at position 16 in 17-oxosteroids.

Steroid	Mol. wt.	$[\alpha]_D$	M_D	Molecular rotation difference
				16α-hydroxy-17-one minus 17-one
K2	304	+11.8	+36	+5 (E)
Dehydroepiandrosterone	288	+10.9 (1)	+31	
16α-hydroxy-androst-4-ene-3:17-dione	292	+194 (2)	+566	+44 (C)
Androst-4-ene-3:17-dione	276	+189 (3)	+522	
16α-hydroxyoestrone	286	+178 (4)	+509	+2 (E)
Oestrone	270	+188 (5)	+507	
				16α-acetoxy-17-one minus 17-one
K2 diacetate	388	+22.9	+89	+76 (E)
Dehydroepiandrosterone acetate	330	+ 3.9 (1)	+13	
3β:16α-diacetoxy-5α-androstan-17-one	390	+57.1 (6)	+223	-6 (C)
3β-acetoxy-5α-androstan-17-one	322	+69 (7)	+229	
16α-hydroxyoestrone diacetate	370	+153 (4)	+566	+108 (E)
Oestrone acetate	312	+147 (8)	+458	

Table 9 contd.

Steroid	Mol. wt.	$[\alpha]_D$	M_D	Molecular rotation difference
3β:16α-diacetoxy-androst-5-en-17-one	388	-18 (9)	-70	-47 (C)
Dehydroepiandrosterone acetate	330	-7 (10)	-23	
				16β-acetoxy-17-one minus 17-one
3β:16β-diacetoxy-androst-5-en-17-one	388	+6 (9)	+23	+46 (C)
Dehydroepiandrosterone acetate	330	-7 (10)	-23	

Abbreviations: E = ethanol C = chloroform

References for Table 9.

(1) Butenandt, A., Dannenbaum, H., Hanisch, G. & Kudszus, H. (1935). Z. physiol. Chemie, 237, 57.

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Table 10. Comparison of some physical properties of K2 diacetate with those of two known 16-acetoxy compounds.

	3β:16β-diacetoxy-androst-5-en-17-one	3β:16α-diacetoxy-androst-5-en-17-one	K2 diacetate
m.p. °C.	165(sinters), 180-181	167-168	167-171
[α]$_D$	+6 ($CHCl_3$)	-18 ($CHCl_3$)	+22.9 (EtOH)

Since the optical rotation of a compound measured in ethanol is more dextro rotatory than when measured in chloroform (Barton and Klyne, 1948) it is not possible to reach any conclusion regarding the configuration of the 16-hydroxyl group from the information available. The melting point evidence would perhaps suggest the 16α-configuration.

It has been shown that 16-oxooestradiol reduces BT as quickly and as strongly as do the C_{21} α-ketols (Touchstone, 1955; Colàs, 1955). It was therefore expected that compounds containing the 16-hydroxy-17-keto grouping would reduce BT in/

in a similar manner and Fried, Thoma, Pearlman, Herz and Borman (1955) have reported that 16α-hydroxyandrost-4-ene-3:17-dione reduced both Tollen's reagent and BT. A small amount of this compound was obtained from Dr Fried and the reduction of BT by 50 μg. of 16α-hydroxyandrost-4-ene-3:17-dione, 50 μg. of 16-oxooestradiol and 25 μg. of 11-deoxycortisol compared. These amounts of steroids were applied to filter paper and sprayed with the BT reagent. 16-Oxooestradiol and 11-deoxycortisol reacted immediately and the intensity of the colour produced by 16-oxooestradiol was about double that produced by 11-deoxycortisol. 16α-Hydroxyandrost-4-ene-3:17-dione reacted slowly and the final colour produced was similar to that given by 3 μg. of 11-deoxycortisol. Thus when the BT reaction is performed on paper the 16-hydroxy-17-oxo group has only 5-10% of the reactivity of the 17-hydroxy-16-oxo group or the 21-hydroxy-20-oxo group. When the BT reaction is performed in solution the reactivity of the 16-hydroxy-17-oxo group appears to be greater since under the alkaline conditions of the reaction the 16-hydroxy-17-oxo structure is partly transformed to the 17-hydroxy-16-oxo structure/

structure (vide Section 11). Considering the above evidence the reduction of BT by purified preparations of K2 could be due to K2 itself and not caused by any contaminant. In this case K2 and Xa would be identical. That neither of the samples of K2 obtained by Colàs reduced BT can be attributed to the fact that the amounts of K2 tested were too small. Of the second sample only 21 μg. were used and this would therefore reduce BT to the same extent as only 1-2 μg. of a C_{21} α-ketol. This amount is at the limit of sensitivity of the reaction for more than 2 μg. per cm.^{2} of a C_{21} α-ketol are needed to give a positive reaction (Bush, 1954).

One difficulty with the acceptance of the structure 3β:16-dihydroxyandrost-5-en-17-one for K2 was the production of formaldehyde on oxidation as previously recorded. When this oxidation was repeated in triplicate on another batch of K2 no formaldehyde was produced. When the residue (weight 8 mg.) from the mother liquors of a K2 crystallization was chromatographed on a 100 x 2 cm. column of Celite using the system benzene: hexane:methanol:water, 6:4:7:3 crystalline K2 was present/

present in those fractions collected between an eluate volume of 960 to 1440 ml. and it was shown by paper chromatography that all the BT reducing material was also present in these fractions.

Thus it was considered that K2 and Xa were identical. It remained to prove the presence of the ketol structure in ring D and to determine the configuration of the 16-hydroxyl group.

11. Sodium borohydride reduction of K2.

The first published report on the use of sodium borohydride as a reducing agent was that of Chaikin and Brown (1949) and since then it has been used quite extensively in the carbohydrate field. It readily reduces aldehydes and ketones, and reductions follow the same pattern as those brought about by lithium aluminium hydride. However it is not quite as active as the latter reagent and thus permits the selective reduction of ketone groups in the presence of other functional groups. Moreover sodium borohydride has the advantage that it can be used in aqueous or methanolic solutions whereas lithium aluminium hydride must be used in non-hydroxylic solvents and protected from moisture. In the steroid field sodium borohydride was/

was first used by Dauben and Eastham (1951) for the reduction of cholestenone enol acetate to cholesterol.

If K2 had the structure 3β:16α-dihydroxy-androst-5-en-17-one then reduction by sodium borohydride should produce androst-5-ene-3β:16α:17β-triol. A sample of K2 (wt. 15 mg.) was reduced by the method described by Norymberski and Woods (1955). To the K2 dissolved in 5 ml. of methanol 6 mg. of sodium borohydride were added and the mixture left at room temperature for 1½ hr. At the end of this time the mixture was diluted with 50 ml. of water, 5.5 ml. N-HCl added and the solution extracted with chloroform. The white residue (14 mg.) obtained from evaporation of the chloroform was crystallized from methanol-benzene to give 13 mg. of crystals. When the melting point was determined in an evacuated tube sublimation of the material occurred and the melting point of the sublimate was much lower than that expected for androst-5-ene-3β:16α:17β-triol. Since the product appeared to be a mixture it was chromatographed on a Celite column using the solvent system benzene:80% methanol. However no separation was obtained and the combined residues/

residues (12.1 mg.) were acetylated. The acetate was crystallized from hexane-ethyl acetate to give white fern-like crystals which melted at 209-210°C. Since the triacetate of androst-5-ene-3β:16α:17β-triol melts at 189-191°C. this compound and the reduction product could not be identical. It could however be the triacetate of androst-5-ene-3β:16β:17β-triol which has been reported to melt at 208-209°. (Huffman and Lott, 1949), 214-215° (Stodola, Kendall and McKenzie, 1941) and 224-226° (Butenandt, Schmidt-Thomé and Weiss, 1939). The weight of the crystalline compound obtained was not determined but it must have accounted for at least 50% of the reduction product.

Thus it could be that the 16-hydroxyl group that it was assumed occurred in K2 had the β configuration. Cooley, Ellis, Hartley and Petrow (1955) have reported that 3β:16β-diacetoxy-androst-5-en-17-one and 3β:16α-diacetoxy-androst-5-en-17-one are converted by standing in alkali to 3β:17β-diacetoxy-androst-5-en-16-one which is the more stable compound. Reduction of a 16-oxo group by sodium borohydride gives a quantitative yield of the 16β-hydroxy compound (Huffman and Lott, 1955). Marrian and Loke (personal communication) have shown/

shown that when 16α-hydroxyoestrone is allowed to stand in normal sodium hydroxide solution for 10 min. 20% is converted to 16-oxooestradiol and this isomerization appears to be complete in about 2 hr. Since solutions of sodium borohydride are alkaline it was possible that if K2 was 3β:16α-dihydroxy-androst-5-en-17-one it was partially isomerized to 3β:17β-dihydroxy-androst-5-en-16-one during the reduction. Reduction of the former compound would produce androst-5-ene-3β:16α:17β-triol and reduction of the 16-oxo compound would produce androst-5-ene-3β:16β:17β-triol. Since the sodium borohydride reduction took place over 1½ hr. and the sodium borohydride was added gradually in small amounts it is quite possible that some isomerization occurred. However Marrian and Loke have also shown that when 16α-hydroxy-oestrone is reduced with sodium borohydride over a period of 1 hr. 80% of an oestriol-like compound and 20% of an <u>epi</u>oestriol-like compound are produced. The minor reaction product has not yet been identified so that it could be either 16-<u>epi</u>oestriol or 17-<u>epi</u>oestriol. Thus the isolated compound resulting from the reduction of K2 could likewise/

likewise be either androst-5-ene-3β:16β:17β-triol or androst-5-ene-3β:16α:17α-triol. Although the melting point of the compound isolated was similar to that of the triacetate of androst-5-ene-3β:16β:17β-triol the melting point of the triacetate of androst-5-ene-3β:16α:17α-triol is unknown and as recorded above the melting points recorded for the triacetate of androst-5-ene-3β:16β:17β-triol vary widely. From the sodium borohydride reduction of K2 therefore it was not possible to draw any conclusions regarding the structure of K2. Proof of the configuration of the 16-hydroxyl group in K2 could best be obtained by catalytic reduction of K2.

12. Catalytic reduction of K2.

In order to complete the identification of K2 a large pool of urine (1300 litres) was fractionated and the amount of K2 obtained, after crystallizing once from methanol, once from benzene and drying to constant weight, was 48 mg. The melting point of this sample of K2 was 176-177°C. when determined on the hot-stage microscope apparatus and 177-181°C. when determined in an evacuated capillary tube.

For the quantitative catalytic reduction of K2/

K2 a Towers micro-hydrogenation apparatus was used. Grasshof (1934) and Butenandt and Dannenbaum (1934) have shown that a double bond in a steroid molecule can be reduced by using a palladium catalyst and that an oxo-group in the same molecule is unaffected. When 10.02 mg. of K2 were reduced in glacial acetic acid using a palladium black catalyst prepared as described by Willstätter and Waldschmidt-Leitz (1921) the corrected hydrogen uptake was 0.719 ml. Assuming K2 to have the structure 3β:16-dihydroxyandrost-5-en-17-one the calculated hydrogen uptake for reduction of the double bond was 0.738 ml. The reduced K2 was isolated by partitioning the acetic acid solution between chloroform and 5% (w/v) sodium carbonate solution. The chloroform was then washed, dried and evaporated as described previously.

It has been shown that using platinum oxide as catalyst both double bonds and ketone groups in the steroid molecule can be reduced (Shoppee,1940; Sarett, 1947). Using a platinum oxide catalyst prepared according to Adams, Voorhees and Shriner (1946) the corrected hydrogen uptake for the hydrogenation of 9.854 mg. of K2 was 1.41 ml. and the calculated value was 1.45 ml. The semi-

reduced/

reduced K2 of the first experiment was hydrogenated using the platinum oxide catalyst and the two samples of fully reduced K2 were combined.

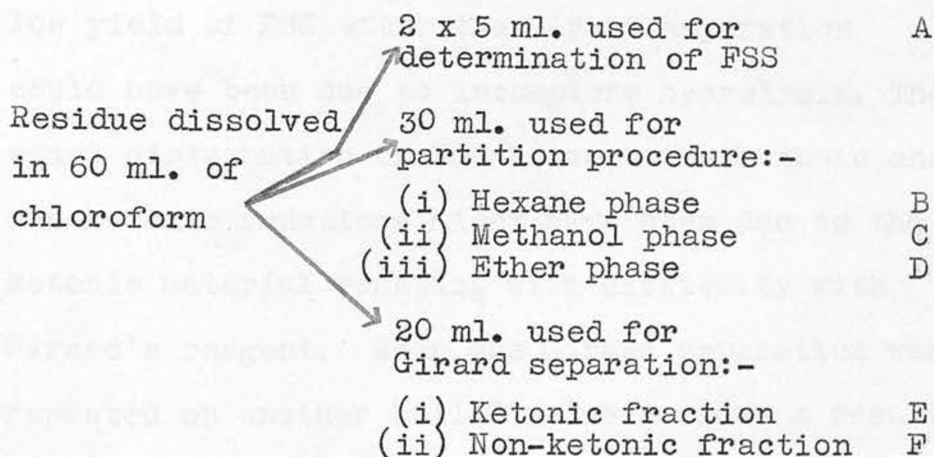
Thus the results of the hydrogenation were in accord with the presence in K2 of a double bond and a ketone group.

The reduced K2 was crystallized from benzene-methanol but the determination of the melting point (220-230°C.) of the crystals obtained suggested that these were impure. The melting point of the expected product, 5α-androstane-3β:16α:17β-triol, is 257-259°C. The crystalline product and its mother liquors were combined (wt. 16.7 mg.) and treated with 3 ml. of anhydrous acetone saturated with hydrogen chloride so that any compound with the hydroxyl groups attached to carbon atoms 16 and 17 in the *cis*-relationship would form an acetonide. After standing at room temperature for 30 min. the acetone solution was diluted with about 10 vol. of ice and water. The insoluble material which separated was filtered off, washed with water and dried. To separate the acetonide, if any, from the unreacted alcohol the residue was leached with benzene (twice with 3 ml.) until there was no further/

further decrease in weight. The residue obtained from evaporation of the benzene weighed 7 mg. The benzene-insoluble material - a white powder - was considered to be impure as judged from its melting point (225-235°C.) and it was therefore acetylated. The acetate was twice crystallized from 66% aqueous methanol and 6.7 mg. of white crystals of melting point 172-174.5°C. were obtained. The melting point of a mixture of the acetate from the reduced K2 and the triacetate of 5α-androstane-3β:16α:17β-triol was also 172-174.5°C. Thus it seemed reasonable to conclude that the hydroxyl group attached to carbon atom 16 had the α configuration so that K2 had the structure 3β:16α-dihydroxyandrost-5-en-17-one.

13. The nature of the formaldehydogenic substances liberated by boiling.

Since Xa and K2 had been shown to be identical it was necessary to enquire into the nature of the formaldehydogenic substances (FSS) liberated by boiling. The residue obtained from a chloroform extract of urine which had previously been extracted and boiled was treated as shown below:-



The residues obtained from fractions B to F were each dissolved in 10 ml. of chloroform and two aliquots of 5 ml. from each fraction used for the determination of FSS. The results are shown in Table 11. Thus while little of the FSS is lost in the partition procedure about half of the FSS is lost during the Girard separation. The Girard separation was performed according to the method described in Section 2 where the Girard hydrazones are/

Table 11. Distribution of FSS liberated by boiling.

	A	B	C	D	E	F
Spekker reading	.203	.000	.124	.432	.098	.099
	.207	.000	.127	.432	.094	.096
FSS (µg. HCHO per litre of urine)	32.7	0	6.6	23.4	8.0	8.0

are only hydrolysed for a period of 2 hr. The low yield of FSS after the Girard separation could have been due to incomplete hydrolysis. The equal distribution of FSS between the ketonic and non-ketonic fractions might have been due to the ketonic material reacting with difficulty with Girard's reagent. When the Girard separation was repeated on another boiled urine residue a result similar to that in the experiment above was obtained. Lengthening the period of hydrolysis by 20 hr. did not release any more FSS. When one half of the non-ketonic fraction was again subjected to the Girard separation no FSS entered the ketonic fraction showing that the ketonic material present in the original extract had no difficulty in forming a hydrazone. The loss of half of the FSS during the Girard separation could be attributed to the acid used for hydrolysis

of/

of the Girard complex.

Two aliquots, each 1/6 of the residue from a chloroform extract of pre-extracted boiled urine, were used for the determination of FSS. The remainder of the residue was divided into two equal parts, each dissolved in 10% aqueous methanol, acidified to pH 1 and allowed to stand at room temperature. One half was extracted after 2 hr. and the other after 20 hr. and duplicate determinations of FSS performed on each extract. The results obtained (Table 12) showed that about half of the FSS released by boiling was destroyed by standing at pH 1 at room temperature for 2 hr.

Table 12. Lability of FSS liberated by boiling.

Time at pH 1 (hr.)	Spekker Readings	FSS (µg. HCHO per litre)
0	.112, .110	17.8
2	.080, .076	10.6
20	.075, .081	10.6

Chromatography of an extract of 5 litres of pre-extracted boiled urine on Celite using the solvent mixture benzene:80% aqueous methanol suggested that most of the FSS released by boiling/

boiling was not steroidal. Nine fractions each containing 50 ml. of eluate were collected and 1/3 of each fraction chromatographed on paper using system B5M. One third of each fraction was also used for the determination of FSS and the values obtained are shown in Table 13. The paper chromatograms showed Compound X to be present in fractions 3 and 4 and the only other BT reducing material present was Compound Y in fractions 4 and 5. Thus the main band of FSS is slightly less polar than Compound X.

Table 13. Chromatography of FSS liberated by boiling.

Fraction number	1	2	3	4	5	6	7	8	9
FSS(µg. HCHO per litre)	0.3	2.4	3.9	2.1	1.2	1.5	1.3	0.1	0

If the major part of the formaldehydogenic material released by boiling i.e. that in fractions 2, 3 and 4, is steroidal then it is possible to make suggestions regarding its structure. Since Compound X, 5β-pregnane-3α:17α:20α-triol and 11-deoxycortisol all have a similar polarity the main band of FSS would be slightly less polar than these compounds. Since the material/

material is formaldehydogenic but not BT reducing an α-glycol side-chain may be present and its release by boiling would suggest that it arose from the sulphate of a steroid with a 5-en-3β-ol group. 3:5-Cyclo-pregnane-6β:20:21-triol would be of similar polarity to X and this structure would be a possible one for the FSS released by boiling. Another possibility would be for the material to be a ring A artifact of a steroid with a 20:21 glycol group or a mixture of such steroids. It is also probable that the released material is not steroidal.

14. Discussion.

It seems certain from the evidence reported here that K2 has the structure 3β:16α-dihydroxy-androst-5-en-17-one (16α-hydroxydehydro*epi*androsterone). K2 and 16α-hydroxyoestrone (G.F. Marrian, personal communication) are the first steroids with the 16-hydroxy-17-oxo group to be isolated from natural sources. Steroids containing this group were unknown until the synthesis of 3β:16α-dihydroxy-5α-androstan-17-one and 16α-hydroxyoestrone di-acetate by Leeds, Fukushima and Gallagher (1954). Since K2 cannot be extracted from unboiled urine by chloroform it must be excreted in a conjugated form/

form. From a consideration of its structure and by analogy with dehydroepiandrosterone (DHA) this conjugated form is almost certainly the 3β-sulphate. Unfortunately the amount of K2 excreted per day is unknown. A minimum value would be about 200 μg. per day. Since hydrolysis of the conjugate of K2 was not complete under the conditions employed and since the intensity of the colour produced by K2 in the blue tetrazolium reaction was less than the intensity of the colour produced by an equal amount of a C-21 α-ketolic steroid such as cortisol or 11-deoxycortisol which has been used as the reference compound it is possible that the amount of K2 excreted per day is greater than the value given above. The fact that K2 has not previously been isolated is no doubt due to the methods of hydrolysing urinary steroid conjugates which have been used. It has been shown in Section 4 that K2 is destroyed by heating with acid. When the method of continuous extraction at pH 1 is used it is probable that some K2 is destroyed by prolonged standing in acid solution in the presence of other urinary constituents or that some K2 is oxidized by the possible presence of peroxides in the ether used for/

for the extraction. The mode of hydrolysis - boiling at neutral pH - used by Dingemanse et al. (1952) would have hydrolysed the conjugate of K2 but these investigators were using the Zimmermann reaction for estimating urinary 17-oxosteroids and since K2 does not react in the Zimmermann test it would not have been detected.

So far few steroids containing the 5-en-3β-ol group have been isolated from urine. Those that have are listed in Table 14[x] which also records some of the physical properties of the compounds. The values given are considered to be the most accurate available and are selected from a collection of all the values reported in the literature both for the isolated and synthetic steroids. Only two, DHA and androst-5-ene-3β:16α:17β-triol, have been isolated from the urine of normal people.

It has been shown that K2 is a metabolite of DHA. When 100 mg. of DHA were administered to an adrenalectomized ovariectomized women about 2% was excreted as Compound X and 1% as Compound Y and none of these compounds were detected in control samples of urine collected before or after the administration of DHA. Structurally K2 is intermediate/

[x] See Appendix II.

intermediate between DHA and androst-5-ene-3β:16α:17β-triol although whether K2 arises by 16-hydroxylation of DHA or by oxidation of the hydroxyl group in position 17 of androst-5-ene-3β:16α:17β-triol is not yet known.

From the urine extracts from which K2 has been isolated androst-5-ene-3β:16α:17β-triol has also been isolated (see Appendix I). This compound has once previously been isolated from normal urine by Marrian and Butler (1944) who employed acid hydrolysis of the urine. The minimum value found for the excretion of the compound was 100 μg. per 24 hr. The amount of this compound detected in the present investigation was also about 100 μg. per 24 hr. and as in the case of K2 this is possibly a minimum value. Thus in addition to DHA normal urine contains two metabolites of DHA with the 5-en-3β-ol group and the amounts of these two metabolites excreted may be quite large. If so the amount of DHA produced per day is greater than is usually measured.

Considered from a quantitative point of view DHA is one of the three most important 17-ketosteroids in normal urine. Prior to 1951 only a fraction of the DHA present in urine was estimated due to the transformations occurring during hydrolysis/

hydrolysis. Recent values suggest that between 1 mg. and 5 mg. of DHA are excreted per day and this accounts for 15% to 30% of the total 17-ketosteroid excretion. The values reported by various authors for normal urine are listed in Table 15. The last column records the method of estimation used. The Pettenkofer reaction and Zimmermann reaction have already been described in this thesis. Those investigators using the Pettenkofer reaction performed this reaction on the ketonic fraction after a Girard separation. Ronzoni used a modified Kober reaction developed by Allen, Hayward and Pinto (1950) and supposed to be specific for DHA and applied this reaction to the residue obtained after continuously extracting urine, acidified to pH 1, for 48 hr. Dingemanse *et al.* separated the 17-ketosteroids chromatographically while Cohen and Oneson used the buffer hydrolysis method. Dobriner performed a digitonin precipitation on the residue from a continuous extraction of urine. For comparison Table 15 also records the daily excretion of androsterone (3α-hydroxy-5α-androstan-17-one) and etiocholanolone (3α-hydroxy-5β-androstan-17-one), the two other 17-ketosteroids present in urine in large amounts. Thus/

Table 15. Amount of dehydro*epi*androsterone present in the urine of normal humans.

Authors	Male or Female	Amount detected mg./24 hr.	% of total 17-ketosteroids	Method of determination
Landau *et al.* (1951)	M and F	1-12.4	10-60	Pettenkofer
Dingemanse, Huis in't Veld & Hartogh-Katz (1952)	M	0.7-8.6	8-41	Zimmermann
	F	0.2-4.9	1-29	
Ronzoni (1952)	M	2.2-5.0	15-26	Allen
Bray & Merivale (1953)	M	4.4±2.4	32.8±8.8	Pettenkofer
	F	4.0±1.5	36.3±8.4	
Prunty (1953)	M	0.5-1.1	8-13	Pettenkofer
	F	0.3-1.4	17-23	
Cohen & Oneson (1953)	M	1.2-2.8 (per litre)	20	Zimmermann
Dobriner (1954)	M	1-2	10-20	Zimmermann
Rubin, Dorfman & Pincus (1954)	M	3.4	28	Androsterone
		3.9	32	Etiocholanolone
	F	3.8	30	Androsterone
		3.0	27	Etiocholanolone

Thus the amount of DHA excreted by females is only slightly less than that excreted by males while it accounts for about the same proportion of the total 17-ketosteroids.

It seems certain now that DHA is derived only from the adrenal cortex and can be used as an indication of adrenal activity (Mason and Engstrom, 1950; Landau, Knowlton, Lugubihl and Kenyon, 1951; Prunty, 1953). There is usually a very large increase in the excretion of DHA in the urine of patients with adrenocortical tumours (Crooke and Callow, 1939). Hirschmann and Hirschmann (1947) isolated 315 mg. per litre of DHA from the urine of a boy with adrenocortical carcinoma and Mason (1948) isolated 613 mg. per litre in another case. In adrenal hyperplasia although the absolute amount of DHA may be increased an abnormally large proportion of the 17-ketosteroids is not excreted in this form (Mason and Engstrom, 1950; Prunty, 1950). In addition the excretion of DHA is usually increased by the administration of ACTH. In the study of Ronzoni (1952) the excretion was approximately trebled in six out of seven cases. Dobriner(1954) found a 3- to 6-fold increase in three out of four cases. Landau <u>et al</u>. (1951) found a 2- to

4-/

4-fold increase and Prunty (1953) a 2- to 10-fold increase although in one case there was no increase. An increased amount of K2 was excreted after the administration of ACTH in the two cases reported in this thesis.

Migeon and Plager (1954) have shown that DHA, probably present as the sulphate, is the most abundant 17-ketosteroid in blood. The amount in peripheral plasma of both males and females was about 40 µg. (Migeon, 1956a). There was a variability of response of plasma DHA to the intravenous infusion of ACTH (Migeon, 1956b). An increase was found 4 to 6 hr. after the beginning of the infusion whereas the 17-hydroxycorticosteroids reached their maximum concentration after 2 hr. It was suggested that the adrenal secretes a steroid other than DHA which was later metabolized to DHA.

While there is much evidence that DHA or its precursor originate in the adrenal cortex it is certain that little or no DHA arises from the testes. DHA is known to occur in the urine of gonadectomized men and women. It is reported not to occur in the urine of patients with an interstitial cell tumour of the testes (Prunty, 1950; Venning/

Venning, 1952a). Landau _et al_. (1951) found no increase in the excretion of Pettenkofer chromogens after the injection of 14,000 i.u. of chorionic gonadotrophin over a 6 day period to two normal young men although their 17-ketosteroid excretion was doubled. DHA excretion is not increased by the administration of testosterone (West, Reich and Samuels, 1951; Dobriner and Lieberman, 1950; Landau _et al_., 1951) and Fukushima, Bradlow, Dobriner and Gallagher (1954) found that after the intravenous infusion of labelled testosterone the DHA isolated from the urine was unlabelled.

Up to the present time DHA has not been isolated from adrenal glands. It would be of interest to determine whether this steroid could be extracted from the adrenals in cases of adrenal tumours. DHA has been isolated from adrenal vein blood of a girl with virilism (Bush, Swale and Patterson, 1956). Bloch, Dorfman and Pincus (1956) have shown that human adrenal gland slices will convert C^{14}-labelled acetate to DHA.

Mason and Kepler (1945a) showed that after giving 1 g. of DHA over 16 days to a man with anterior pituitary insufficiency the DHA isolated from urine constituted 20% of the 17-ketosteroids isolated; they also isolated 20% as 3α-hydroxy-

5β-/

5β-androstan-17-one, 35% as 3α-hydroxy-5α-androstan-17-one and 1% as androst-5-ene-3β:17β-diol. Similar results have also been reported by Mason and Kepler (1947), Miller, Dorfman and Miller (1950), and Landau et al. (1951). Ungar, Miller and Dorfman (1954) isolated DHA, 3α-hydroxy-5β-androstan-17-one, 3α-hydroxy-5α-androstan-17-one and 3α:17β-dihydroxy-5β-androstane from the urine of a man who received androst-5-ene-3β:17β-diol. Dorfman (Dorfman and Shipley, 1956) has reported that 3β:17α-dihydroxy-pregn-5-en-20-one administered to humans is converted to DHA whilst Lieberman (1956) obtained no evidence for this reaction *in vivo*. Since no details have been published of either of these experiments it is not possible to arrive at any conclusion.

Thus except for DHA, androst-5-ene-3β:17β-diol and ACTH no other substance increases DHA excretion. If endogenously produced DHA is metabolized in the same way as administered DHA a large amount of the androsterone and etiocholanolone in urine must come from DHA and DHA and its derivatives would thus account for the major portion of 17-ketosteroids of adrenal origin.

The/

The results from <u>in vitro</u> studies have agreed with those obtained <u>in vivo</u>. Schneider and Mason (1948) found that DHA incubated with rabbit liver slices was converted to androst-5-ene-3β:17β-diol and androst-5-ene-3β:16α:17β-triol. Ungar, Miller and Dorfman (1954) showed that perfusion of DHA through rat livers produced androst-5-ene-3β:17β-diol and androst-4-ene-3:17-dione. After perfusion of DHA through beef adrenals 11β-hydroxy-androst-4-ene-3:17-dione was isolated (Meyer, Jeanloz and Pincus, 1953), and following the incubation of DHA with bovine adrenal homogenates Meyer, Hayano, Lindberg, Gut and Rodgers (1955) detected 28 different compounds. One half of the DHA was unmetabolized and other steroids with a 5-en-3β-ol group, if present, were present in only trace amounts. Androst-4-ene-3:17-dione and 11β-hydroxy-androst-4-ene-3:17-dione were the main metabolites. Samuels, Helmreich, Lasater and Reich (1951) have shown that endocrine glands that produce steroids contain an enzyme 3β-ol-dehydrogenase which converts the 5-en-3β-ol group of steroids to the 4-ene-3-keto group. From the work of Ungar, Miller and Dorfman (1954) it appears that this enzyme also occurs in rat livers.

The/

The metabolism of DHA, resulting from _in vivo_ and _in vitro_ studies, has been summarized by Dorfman (1953). Some is excreted unchanged whilst a greater amount is converted to androst-4-ene-3:17-dione which in turn is metabolized to androsterone and etiocholanolone. Small amounts of DHA are converted to androst-5-ene-3β:16α:17β-triol and possibly trace amounts are converted to androst-5-ene-3β:17β-diol. The present work has shown that K2 is an important metabolite of DHA.

Although the metabolism of DHA is now well known little is known concerning the origin of DHA.

Marker (1938, 1941) suggested that the 5-en-3β-ol group arose from the 4-en-3-one group but evidence from experiments in which steroids with the 4-en-3-one group have been administered to people would seem to disprove this suggestion. The main metabolic reaction for the 4-en-3-one group is reduction to the saturated alcohol. However Munson, Labhart and Forsham (1952) reported that the administration of 100 mg. per day of cortisol or cortisone to three adrenalectomized gonadectomized men increased the amount of Pettenkofer chromogens excreted. After the administration of/

of cortisone the DHA excreted accounted for 40% of the urinary 17-ketosteroids but after administration of cortisol the figure was only 6%. These findings are hard to accept for so far there is no evidence that an oxygen atom at position 11 in the steroid molecule can be removed. It is also known that in congenital adrenal hyperplasia administration of cortisone reduces the excretion of 17-ketosteroids and DHA (Gardner and Migeon, 1952). Wolfson, Eya and Robinson (1952; Wolfson, 1954) also suggest that the 5-en-3β-ol group arises from the 4-en-3-one group and further suggest that corticosterone is the major source of Pettenkofer chromogens in urine. Although the excretion of DHA was not increased by injection of corticosterone the excretion of three other Pettenkofer chromogens, of unknown nature, was.

DHA could be derived directly from cholesterol. Hirschmann and Hirschmann (1945, 1947) have suggested that the reaction between 17-ketosteroids and C-21 steroids can proceed in both directions and that DHA may be formed from cholesterol without passing through a C-21 intermediate. Thus in cases of adrenal tumours the/

the conversion of cholesterol to DHA is supposed to proceed at a rate that is greater than the conversion of DHA to other compounds so that DHA is released in large amounts from the adrenal cortex. That the large amounts of DHA found in the urine of patients with adrenal tumours is not due to saturation of the enzyme systems involved in its conversion has been shown by Mason and Kepler (1947). When 1.2 g. of DHA was administered over eight days to a woman with an adrenal tumour and whose 17-ketosteroid excretion averaged 250 mg. per day there was only a small increase (19 mg.) in the average 17-ketosteroid excretion and no change in the pattern of steroid excretion.

Another possible route for the synthesis of DHA would involve a direct synthesis from acetate without cholesterol being an intermediate. Such a possibility has been suggested by Brady (1951) and by Stone and Hechter (1954) but more evidence is needed to support this scheme.

Since cholesterol is present in the adrenal in large amounts and since it has the 5-en-3β-ol group which occurs also in DHA it has received most attention as a precursor of DHA. The scheme for which most evidence is available involves the conversion of cholesterol to a C-21 steroid which then/

then has the side-chain removed. It is known from the use of radio-active cholesterol _in vivo_ and _in vitro_ that cholesterol is a precursor of the C-21 steroid hormones (see Hechter and Pincus, 1954). The scheme postulated by Hechter and Pincus for the biosynthesis of corticosteroids is shown in Fig.14. Pregnenolone (3β-hydroxy-pregn-5-en-20-one) has now been isolated from cow adrenal homogenates incubated with cholesterol (Saba, Hechter and Stone, 1954). Since there is no evidence that

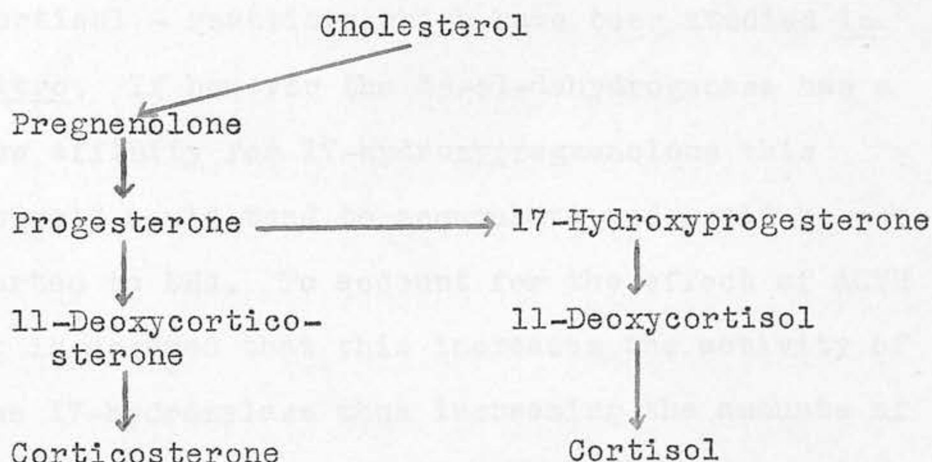


Fig. 14. Scheme of corticosteroid biosynthesis.

17-deoxy C-21 steroids can be converted to C-19 steroids the pregnenolone formed would have to undergo 17-hydroxylation before it could be converted/

converted to DHA and an active 17-hydroxylase is known to occur in the adrenal. Removal of the side-chain would give DHA. This is the scheme suggested by Lieberman and Teich (1953).

It is possible that there are two main pathways for the pregnenolone that is produced in the adrenal. The main one is its conversion to progesterone by the enzyme 3β-ol-dehydrogenase; the second is hydroxylation by the enzyme 17-hydroxylase. The progesterone produced is then hydroxylated at C-17 and further hydroxylated to cortisol - reactions which have been studied _in vitro_. If however the 3β-ol-dehydrogenase has a low affinity for 17-hydroxypregnenolone this steroid would tend to accumulate and could be converted to DHA. To account for the effect of ACTH it is assumed that this increases the activity of the 17-hydroxylase thus increasing the amounts of 17-hydroxyprogesterone and 17-hydroxypregnenolone formed. In cases of adrenal tumours it is necessary to postulate that the cells, which appear to be autonomous, lack the 3β-ol-dehydrogenase or have an extremely active 17-hydroxylase. The cells of adrenal tumours do not appear to differ histologically in most cases from normal adrenocortical cells/

cells (Cahill, 1944).

With regard to the action of ACTH Stone and Hechter (1954) suggested that this hormone increases the conversion of cholesterol to pregnenolone. However there is some evidence that the enzyme 17-hydroxylase may also be affected. After hypophysectomy the zona glomerulosa appears to remain normal while the zona fasciculata atrophies and after the administration of labelled ACTH the labelling is localized in the zona reticularis. Ayres, Gould, Simpson and Tait (1956) have shown that there is a differential production of corticosteroids in the ox adrenal gland. The zona fasciculata contained mainly cortisol and little aldosterone whereas the zona glomerulosa contained more aldosterone than cortisol. Kass, Hechter, Macchi and Mou (1954) found that the predominant corticosteroid in the adrenal vein blood of rabbits treated for 21 to 28 days with ACTH was cortisol whereas non-treated rabbits secrete corticosterone. In this case ACTH had changed the secretion from the 17-deoxy to the 17-hydroxy type of steroid. Hechter and Pincus (1954) have reported that the steer adrenal produces/

produces primarily corticosterone and little or no cortisol and that the ability of the perfused steer adrenal to 17-hydroxylate progesterone is absent or minimal. Macchi and Hechter (1954) found that perfused steer glands either failed or responded poorly to ACTH although the corticosteroid production of perfused cow adrenals was greatly increased by the addition of ACTH to the perfusion fluid. It does appear therefore that ACTH has some effect on the enzyme 17-hydroxylase.

Since the gonads and the adrenal cortex are derived from the same embryonic tissue it is probable that the first steps in the synthesis of the hormones of the different glands are the same. Slaunwhite and Samuels (1956) have shown that testes slices will convert progesterone to testosterone and that 17-hydroxyprogesterone is an intermediate. An alternative pathway would be via 17-hydroxy pregnenolone and DHA. However if there is present in testes a large amount of 3β-ol-dehydrogenase compared to the amount of 17-hydroxylase then almost all the pregnenolone formed would be oxidized to progesterone. If this scheme is correct it could be that gonadotrophin acts to accelerate the last stage of the synthesis - the reduction/

reduction of androst-4-ene-3:17-dione to testosterone (17-hydroxy-androst-4-en-3-one). The equilibrium *in vivo* between 17-ketosteroids and C-19 17-hydroxysteroids favours very much the 17-ketosteroids.

If the synthesis of steroids takes place by the same route in all steroid producing tissues then it would be expected that steroids which are intermediates in the synthetic process, or compounds derived from these, would be common to all the steroid producing organs. Thus progesterone has been isolated from ovaries, adrenals and placentae, 3β-hydroxy-5α-pregnan-20-one from ovaries, adrenals, placentae and testes and 3α-hydroxy-5α-pregnan-20-one and 3β-hydroxy-pregn-5-en-20-one from testes.

The schemes proposed above suggest that DHA and the other 5-en-3β-ol steroids have no physiological activity and are merely intermediates, or derived from intermediates, in the synthetic process. DHA is weakly androgenic being about half as active as androsterone (Fieser and Fieser, 1949). It has no effect on nitrogen retention even when administered in large doses (Mason and Kepler, 1945a) nor has androst-5-ene-3β:17β-diol (Albright/

(Albright, 1942). Pregnenolone is active with respect to spermatogenesis and prevents the testicular atrophy that normally follows hypophysectomy (Albert and Selye, 1942; Masson, 1945). Polley and Mason (1950) did not observe any biological effects when pregnenolone and 21-hydropregnenolone were administered to humans with rheumatoid arthritis. Pregnenolone taken by mouth has been reported to enhance skilled psychomotor performance and to be more active in this respect than cortin (Pincus and Hoagland, 1944). Whether or not DHA has any activity in the foetus is an interesting possibility. Read, Venning and Ripstein (1950) showed that large amounts of 17-ketosteroids were excreted in the first two days of life followed by a downward trend. Migeon (1956 c) has confirmed these results and found that at six weeks of life the plasma DHA level was 8-12 μg.% and that of androsterone 9 μg.%. These levels had fallen to zero after six months. The maternal DHA levels were lower than the levels in cord plasma and lower than the average for normal non-pregnant females.

Considering the above facts it is not expected that K2 will have any physiological activity/

activity. The finding of Speirs _et al_. (1951) that boiling urine without loss of volume increased its activity compared with unboiled urine in the eosinophil test will need reinvestigation.

PART II.

COLD ACID-LABILE FORMALDEHYDOGENIC SUBSTANCES IN HUMAN URINE.

1. Introduction.

It is well known that many steroids are transformed by being heated in acid solution. This part of the thesis is concerned only with substances which are labile to cold acid, i.e. transformed by standing in acid solution at pH 1 at room temperature. The method of estimation used would detect those substances which give rise to formaldehyde after oxidation with periodic acid. Of the steroids only those containing a ketol or glycol grouping at positions 20 and 21 are so oxidized.

Paterson and Marrian (1953b) reported that formaldehydogenic substances (FSS) occur in normal human urine which lose their formaldehydogenic properties on standing at room temperature in acid (pH 1) solution for two hours. The increased amounts of cold acid-labile FSS found in the urine of a patient injected with adrenocorticotrophic hormone (ACTH) suggested that such FSS probably originated in the adrenal cortex. The work described below was commenced in order to discover further properties of this FSS/

FSS. However, the presence of cold acid-labile FSS in urine was not confirmed and it appeared that most, if not all, of the labile FSS detected by Paterson and Marrian originated in the solvents used for the extraction of urine.

Heard, Sobel and Venning (1946) observed that when urine was acidified the amount of reducing substances which could be extracted by a chloroform-ether mixture was greater than that obtained when the extraction was performed at neutral pH. Sixty per cent. of the reducing substances present were destroyed by standing for one hour at room temperature with 2 vols.% of concentrated sulphuric acid. When urine was neutralized immediately after acidification and then extracted the amount of reducing substances extracted was the same as that extracted from unacidified urine. Paterson, Cox and Marrian (1950) found that preliminary acidification also increased the amounts of formaldehydogenic substances which could be extracted from urine and these authors were the first to indicate the presence in human urine of FSS which was labile to cold acid. If urine, acidified to pH 1, was allowed to stand at 25° and aliquots withdrawn at various time intervals for the/

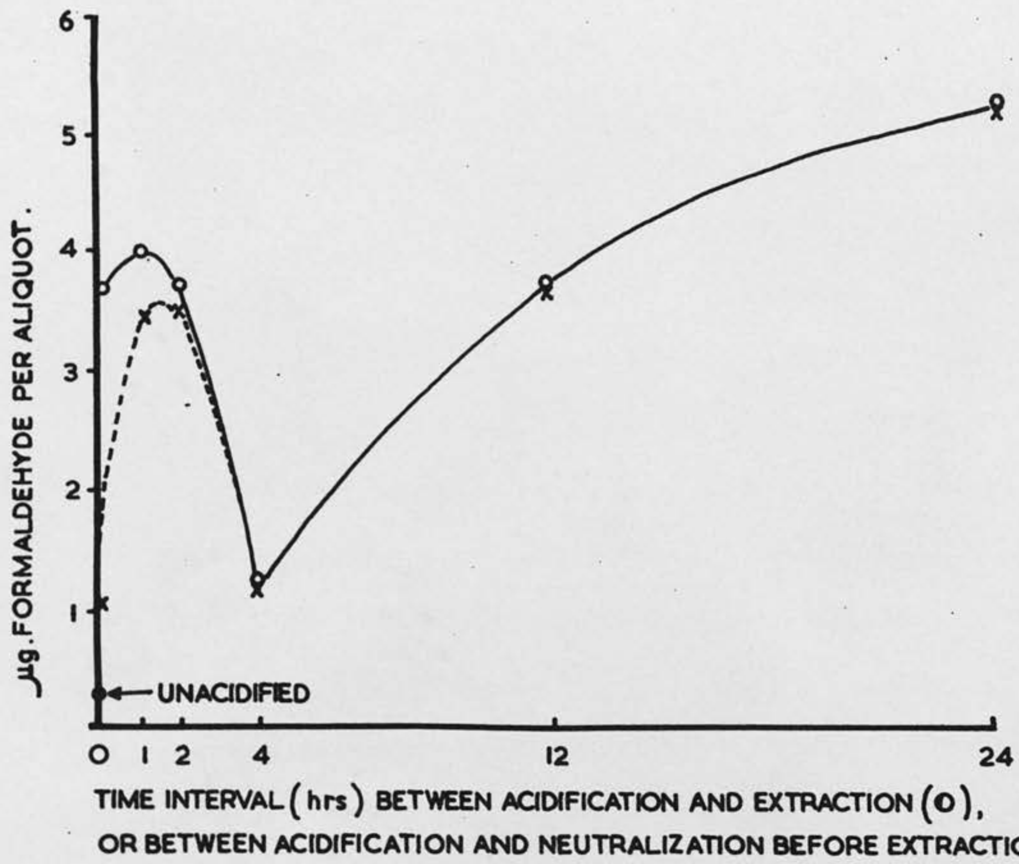


FIG. 15. HYDROLYSIS CURVE OF URINE.

the determination of FSS then hydrolysis curves of the type shown in Fig. 15 were obtained. If the urine was neutralized immediately before extraction the values of FSS obtained are shown by crosses in Fig. 15. The results could be interpreted to mean that there were two groups of conjugated FSS; the ionization of one group - the chloroform soluble conjugates - was suppressed at pH 1 and the unionized form was thus extracted by chloroform. When the urine was at neutral pH the conjugate was ionized and not extractable with chloroform. Since the effect of neutralization decreased with time it was supposed that complete hydrolysis occurred within the first two hours of acidification and the decrease in the amount of FSS extracted between two and four hours was due to destruction by acid of the liberated FSS. This conjugate was shown not to be a glucuronidate (Paterson and Marrian, 1951). The other group of conjugates - the chloroform insoluble conjugates - was not extracted from acidified urine by chloroform. The slow rise after four hours in the amount of FSS extracted from acidified urine was due to a slow liberation of FSS from chloroform insoluble conjugates. Further studies by Cox and/

and Marrian (1951) on this latter group of conjugates showed that when subjected to the action of a β-glucuronidase preparation two different types of compounds were released. One type was slowly destroyed at pH 1 at room temperature whereas the other type was stable.

In the experiments reported above the chloroform extracts of urine were not washed with either alkali or water and Paterson and Marrian (1953a) have shown that such extracts will react with and retain added formaldehyde. Alkali-washed extracts of urine also have this property to a lesser extent and the formaldehyde retaining power may be increased by acid hydrolysis of urine at room temperature. When alkali-washed extracts of urine were used the presence of cold acid-labile FSS was not always indicated and there was a possibility that any apparent destruction of FSS was due to formaldehyde retention. To test this possibility methods of determining formaldehyde retention were elaborated by Paterson and Marrian (1953b) and experiments were performed on the same urine specimen to determine (1) the formaldehyde retaining capacity, (2) the FSS by direct oxidation with periodic acid, and (3) the FSS after formaldehyde/

formaldehyde pretreatment at varying time intervals after acidification to pH 1. When corrected for formaldehyde retention it appeared that there was a destruction of FSS of between 20% to 30% during the first two hours after acidification.

It was also shown that the cold acid-labile FSS was present in the unconjugated fraction rather than liberated by hydrolysis of conjugates. Acidification of chloroform extracted urine increased the amount of extractable FSS whereas acidification of the chloroform extract of urine caused a destruction of FSS which was complete in about one hour. Only a part of the total unconjugated FSS in urine was labile at pH 1 and room temperature. Values for cold acid-labile FSS ranged from 8.4 to 27.4 µg. formaldehyde per 24 hour.

Although nothing was known of the nature of this cold acid-labile material it was assumed to be steroidal and to originate in the adrenal cortex. In an experiment on a rheumatoid arthritic man injected with ACTH the excretion of the cold acid-labile FSS was increased and its excretion pattern was similar to that of the cold acid-stable unconjugated FSS and the hot acid-stable FSS, which are probably artifacts formed from/

from the C_{21} 17-hydroxy-corticosteroids, and hot acid-stable acetaldehydogenic substances.

2. Methods used.

Two methods of determining cold acid-labile FSS have been used.

Method 1.

This method was used by Paterson and Marrian. One third of a 24 hr. specimen of urine was extracted twice with 2 vols. of chloroform. The combined chloroform extracts were washed twice with 1/5 vol. of 5% sodium bicarbonate solution, twice with 1/5 vol. of water, dried for about 10 min. over anhydrous sodium sulphate A.R. and evaporated to dryness under reduced pressure in a water bath at a temperature of less than 40° C. The residue was dissolved in 2 ml. ethanol, diluted to 100 ml. with water and divided into two equal parts. Both parts were acidified to pH 1 by adding 5.5 ml. of $N-H_2SO_4$. One part was extracted immediately after acidification and the other after standing at pH 1 for 1 hour. Each part was extracted twice with 2 vols. chloroform and the chloroform extracts washed, dried and evaporated as described above. The residue was dissolved /

dissolved in 1.5 ml. ethanol and two 0.5 ml. portions oxidized for the determination of FSS. In later experiments, to avoid the use of ethanol, the final dried chloroform extract was divided into two equal parts and each part evaporated to dryness. Each residue was transferred quantitatively by the use of chloroform to a distillation flask (approximately 30 ml. capacity), the chloroform evaporated _in vacuo_ and the last traces of chloroform removed by leaving the flask in an evacuated desiccator for 10 min. Each residue was then dissolved in 0.2 ml. glacial acetic acid.

Method 2.

A washed and dried chloroform extract of urine was prepared as described above and divided into two equal parts. To each part 20 vols.% ethanol and 1 vol.% 5M-H_2SO_4 were added, one part being washed immediately with alkali and water and the other after standing for one hour at room temperature. After drying each extract was divided into two equal parts and evaporated to dryness _in vacuo_ at a temperature less than 40°C. The residues obtained were transferred to distillation flasks with chloroform as described above.

Oxidation/

Oxidation procedure, distillation of formaldehyde and colour reaction.

These were performed as described by Paterson and Marrian (1953a). To the urinary residue dissolved in ethanol or acetic acid was added sufficient water to make the volume up to 1 ml., 2 ml. of a solution of HIO_4 in 0.4 N-H_2SO_4 were added and the oxidation allowed to proceed at room temperature for two hours. The oxidation was stopped by the addition of 3 ml. 28% $ZnCl_2$ in 1.2 N-HCl. After adding 3 ml. 10N-H_2SO_4 the oxidation mixture was distilled into a test tube graduated at 5 ml. and 7 ml. and containing 1 ml. of 4% aqueous Na_2SO_3 solution. During distillation the tip of the condenser was immersed in the distillate. Distillation was stopped when the volume of liquid in the receiver was 5 ml. and after washing down the tip of the condenser the distillate was diluted to 7 ml. with water. Three ml. of this solution were transferred to a test tube, 5 ml. of 0.2% chromotropic acid in 30N-H_2SO_4 added and the mixture heated in a boiling water bath for 30 min. After cooling to room temperature the mixture was diluted to 10 ml. with 18N-H_2SO_4 and the intensity of the colour developed measured/

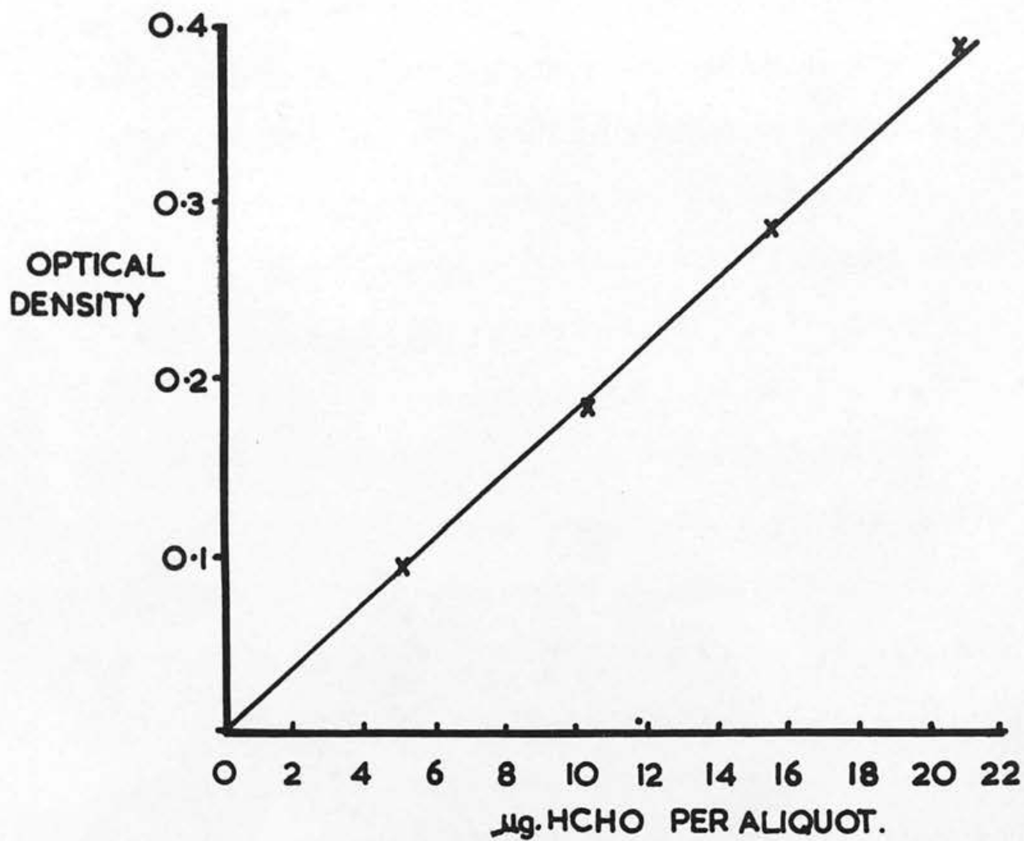


FIG.16. CALIBRATION CURVE FOR FORMALDEHYDE ESTIMATIONS.

measured in a 'Spekker' photoelectric absorptiometer, using Ilford 605 filters, against a reagent 'blank' mixture.

Calibration curve.

A specimen calibration curve is shown in Fig.16. This was prepared from a standard solution of hexamethylene tetramine containing 16.3 μg. per ml. This is equivalent to 20.9 μg. formaldehyde per ml. Aliquots of this solution were used and the volume made up to 1 ml. with water. Stannous chloride and sulphuric acid were added and the procedure described above was followed.

Values for the FSS determined are expressed as μg. formaldehyde per 24 hr. measured after periodate oxidation rather than as mg. per 24 hr. of some hypothetical steroid.

3. Properties of cold acid-labile FSS.

Using method 1 the presence in normal male urine of FSS labile to cold acid in amounts varying between 8 μg. and 20 μg. HCHO per 24 hr. was readily confirmed. When method 2 was evolved it was necessary to check these values. Method 2 was much quicker than method 1 in that the second extraction/

extraction of FSS, from aqueous ethanol, was dispensed with. Also the method should be more quantitative and accurate since the volume of chloroform used is much greater than that used in the second extraction in method 1. Values for cold acid labile FSS from 0 to 19.2 µg. HCHO per 24 hr. were obtained.

The time required for destruction of the cold acid-labile FSS was also determined. A washed and dried chloroform extract of urine was divided into five equal parts and determinations of FSS were performed on each part after they had stood for varying intervals of time at pH 1 at room temperature. The results recorded in Table 16 confirmed that destruction of the FSS was complete in ½ hr. to 1 hr.

Table 16. Time required for destruction of cold acid-labile FSS.

pH 1 hr.	Urine 1		Urine 2		Urine 3	
	Spekker reading	FSS	Spekker reading	FSS	Spekker reading	FSS
0	0.060 0.065	45.0	0.053 0.055	52.5	0.052 0.052	51.0
½	0.047 0.048	34.9	0.040 0.040	39.0	0.040 0.042	40.5
1	0.040 0.045	31.5	0.040 0.038	38.2	0.045 0.043	42.0
2	0.040 -	29.3	0.043 0.039	40.5	0.042 0.042	41.2
3	0.042 0.039	29.3	0.038 0.042	39.0	0.041 0.040	39.7

FSS in µg. HCHO per 24 hr.

In all the experiments performed so far the chloroform extracts had been evaporated at less than 40°C. Experiments were performed to determine whether the cold acid-labile FSS were also thermolabile. Washed and dried chloroform extracts of urine/

urine were divided into two equal parts and cold acid-labile FSS determined in each. The chloroform of one half was evaporated to dryness at a temperature of less than 40°C.; the other chloroform extract was evaporated at 60°C. or refluxed for one hour prior to evaporation. The results, recorded in Table 17, suggested that the cold acid-labile FSS was not thermolabile.

Table 17. Thermostability of cold acid-labile FSS.

	Evaporated at 40°C.		Refluxed at 60°C.	
	Total FSS	Acid-labile FSS	Total FSS	Acid-labile FSS
Urine 1	34.1	12.8	34.1	12.8
" 2	36.0	0	32.4	0
" 3	26.4	0	30.4	2.0
" 4	21.2	0.8	17.6	0

FSS in μg. HCHO per 24 hr.

Many of the experiments reported above were performed not on 24 hr. samples of urine but on combined 3 hr. collections of urine so that there would be less chance of any acid-labile material undergoing/

undergoing change during collection of the urine.

4. FSS present in solvents.

Soon after the commencement of the work it was discovered that the ethanol and chloroform used in the experiments contained considerable amounts of FSS. The alcohol used had been purified by allowing it to stand over m-phenylenediamine for several days and then distilling. Determinations by Mrs Atherden showed this ethanol to give rise to about 1 μg. HCHO per ml. on periodate oxidation. Ethanol almost free of FSS could be obtained by the following procedure: (i) distil, (ii) oxidize for 1 hr. with periodic acid, (iii) decompose excess periodate with stannous chloride, acidify and distil and (iv) allow distillate to stand over m-phenylenediamine for four days and redistil. Since the FSS in ethanol could not be entirely removed and since it appeared that it was also capable of being regenerated the use of ethanol was dispensed with in later experiments and glacial acetic acid utilized. The FSS in chloroform was more difficult to eliminate; a varying proportion of it/

it was shown to be acid-labile but since the purification by acid treatment was time-consuming and costly and only partially successful it became necessary to do blank determinations on the chloroform used in each experiment. Marrian and Atherden (1953) found the FSS in chloroform to give 1.0-1.2 μg. HCHO per litre. More recent determinations have shown the value to vary between 1 and 3 μg. HCHO per litre, so that for a 24 hr. collection of urine the FSS in the chloroform used in its extraction could account for between 6 and 18 μg. HCHO per 24 hr. Using the above values for FSS in ethanol and chloroform it is possible to calculate the amount of FSS contributed by the solvents to the total FSS determined in an experiment of the type described by Paterson and Marrian (1953b). Assuming a urine volume of 1500 ml., then as much as 30 μg. HCHO per 24 hr. could be obtained from FSS in the solvents used. That large amounts of FSS were present in the solvents used is suggested by the report of Paterson (1953) that about 80% of the FSS were found in the first chloroform extract of urine and about 15% in the second. These results have been confirmed by Mrs Atherden/

Atherden and these experiments also showed that the FSS measured in the second $CHCl_3$ extract are no greater than the FSS found in an equivalent volume of chloroform treated in the same way. Thus approximately 30% of the FSS determined by Paterson must have come from the solvents used.

Some of the FSS in chloroform were shown to be acid-labile. Cold acid-labile FSS were determined in large volumes of chloroform (4 to 6 litres) using method 1. The results are recorded in Table 18.

Table 18. Determination of cold acid-labile FSS present in chloroform.

Time stood at pH 1	Experiment 1		Experiment 2	
	Spekker reading	FSS	Spekker reading	FSS
0 hr.	0.007 0.007	1.2	0.020 0.021	2.2
1 hr.	0.002 0.003	0.4	0.015 0.014	1.6

FSS in the solvents were not affected by being refluxed for one hour.

The/

The discovery of so much FSS in the solvents used and that fact that some of it was cold acid-labile raised doubts concerning the existence of cold acid-labile FSS in urine. Accordingly a series of urines were assayed for cold acid-labile FSS and with each determination a chloroform blank was included. Although, at varying times, fourteen determinations of cold acid-labile FSS were made on normal male urines in only two cases were significant amounts (i.e. greater than 6 μg. HCHO per 24 hr.) found. For most determinations method 2 was used; it has been shown that the ethanol used to dissolve the acid in chloroform contributed little, if any, FSS to the total.

The opportunity arose of studying the urinary excretion of FSS of a female patient on a salt-free diet. After a control period of ten days the patient was on the diet for twelve days. Cold acid-labile FSS were determined on twenty days using method 2. On only four days were significant amounts (more than 6 μg. HCHO per 24 hr.) of cold acid-labile FSS detected.

5. /

5. <u>Cold acid-labile FSS in the urine of normal males after injection of ACTH.</u>

If the cold acid-labile FSS was an artifact it would not be expected to increase after the injection of ACTH. Paterson and Marrian (1953b) had found amounts of cold acid-labile FSS of 40 μg. HCHO per 24 hr. in the urine of a patient with rheumatoid arthritis injected with ACTH. It was therefore essential to test the effect of ACTH on the excretion of FSS and two experiments were performed on normal males. After a three day control period 40 units of 'ACTHAR' gel were injected daily for two days and the determinations continued for a further three days. In the first experiment cold acid-labile FSS were determined by both methods and chloroform 'blanks' were performed on each pool of chloroform used. Although after the injection of ACTH the excretion of unconjugated FSS increased four-fold only insignificant amounts (0 to 2.8 μg. HCHO per 24 hr.) of cold acid-labile FSS were detected.

In the second experiment only method 1 was used and on most days the effect of evaporating the/

the chloroform extract at 60°, or refluxing it for ½ hr. before evaporating, was determined. The increase in FSS after the injection of ACTH was smaller than in the first experiment (100% increase) and again only insignificant amounts of cold acid-labile FSS (0 to 3.3 μg. HCHO per 24 hr.) were detected. Evaporating at a higher temperature or refluxing did not affect the results. Thus the finding of Paterson and Marrian was not substantiated.

6. Discussion.

The occurrence of cold acid-labile FSS in urine has also been reported by Bayliss (1952) and Venning (1952b; Venning, Dyrenfurth and Kazmin, 1953). Venning found that extraction of urine at pH 1 at frequent intervals over 24 hours gave a higher value than an extraction on acidification to pH 1 and another extraction 24 hours later using both chemical and bioassay. This does not necessarily mean that destruction due to acid was taking place; it could be that frequent removal of the hydrolysis products allows further hydrolysis to proceed and that this is inhibited if the products/

products are not removed, or that frequent removal prevents destruction by other means, e.g. by microorganisms. More direct evidence for the presence of acid-labile material was obtained from an experiment in which two lots of urine were allowed to stand at pH 1 for 3 hr. and for 24 hr. The urines were extracted first at pH 1 and the sodium hydroxide washings were analysed. The results are shown in Table 19.

Table 19. Effect of acid on recovery of corticoids. Two lots of urine acidified to pH 1.

	A. Extracted after 3 hr.			B. Extracted after 24 hr.		
	FSS	Bioassay	PS	FSS	Bioassay	PS
Extracted at pH 6.	0.633	0.042	0.076	0.855	0.025	0.113
Re-extracted at pH 1	0.367	0.033	0.029	0.317	0.032	0.044

Quantities expressed in mg. of steroid per litre of urine.

PS = substances giving a positive reaction in the test of Porter and Silber (1950); i.e. of steroids those with a dihydroxyacetone side-chain.

Although/

Although there was an increased amount of corticoid extracted at pH 6 when chemical procedures were used there was a loss of activity judged by bioassay. These two experiments on whole urine are difficult to evaluate because the two processes of destruction and hydrolysis are taking place at the same time and urine may contain formaldehydogenic material which is not steroidal. The decrease in material found by bioassay would, if it were a formaldehydogenic steroid, correspond to about 1.5 µg. HCHO per litre of urine which is very much less than the amounts of labile material detected by Paterson and Marrian. Also before it is assumed that the labile FSS, if present in urine, and the material detected by Venning are the same it must be shown that the destruction in Venning's experiments also occurs in ½ - 1 hr. after acidification. In the bioassay performed by Paterson (unpublished) using the liver glycogen test the activity of the chloroform extract of urine was increased by standing at pH 1 for 3 hr. It must also be shown that the decrease found by bioassay is accompanied by a decrease in FSS. The decrease in the bioassay/

bioassay value could be due to a transformation of some part of the molecule other than the side chain. Venning has also found conjugated corticoids which are soluble in chloroform and which are removed by alkali washing. Some of these conjugated corticoids are labile at pH 3 as judged by bioassay. It is unlikely that this material is the same as the labile FSS detected by Paterson and Marrian for in the experiments of the latter authors such material would have been removed by the washing procedure employed. The amount of labile corticoid found by Venning (0.060 mg. per 24 hr.) represents about 5 μg. HCHO if formaldehydogenic.

Bayliss (1952) has attempted to study the different types of conjugated FSS in urine and to devise a procedure that would give the maximal yield of hydrolysed FSS. He confirmed that with most urine specimens extraction immediately after acidification to pH 1 gave a higher yield of FSS than extraction at pH 7.4 but found no significant difference between FSS obtained from urine extracted at pH 1 and that obtained by first extracting at pH 7.4 followed by re-extraction at pH 1. It was/

was shown that most of the FSS extracted from urine at pH 1 was acid-labile when stood at pH 1. at room temperature for 24 hr. but no experiments were performed to determine whether the destruction was gradual or complete within one hour. Other experiments to determine the effect of prolonged standing in acid on the yield of FSS used whole urine. A gradual destruction occurred over 24 hr. (in contrast to the finding of Paterson, Cox and Marrian,1950) and in some cases the whole of the unconjugated fraction was destroyed. In these experiments little destruction occurred during the first four hours; most occurred between 4 hr. and 24 hr. after acidification.

De Courcy and Gray (1953) have also studied the effect of acid on urinary FSS. They have shown that the amount of formaldehyde liberated when washed urinary extracts are dissolved in glacial acetic acid and oxidized is greater than when the extracts are dissolved in ethanol and oxidized under the same conditions. Paterson and Marrian (1953a) found that the same values for FSS were obtained for the same extract with either solvent/

solvent. Although de Courcy and Gray could obtain acid hydrolysis curves similar to those of Paterson, Cox and Marrian when using the method of Talbot, Saltzman, Wixon and Wolfe (1945) for measuring reducing steroids, no regular form of hydrolysis curve was obtained when the formaldehydogenic method was used. Frequently there was no change in the amounts of FSS determined at varying times of hydrolysis. Using paper chromatography and spray reagents which would detect steroids with a Δ^4-3 keto group or an α-ketol group it was found that after urine had been extracted at neutral pH extracts prepared by extraction immediately after acidification to pH 1 or after standing at pH 1 for 24 hr. rarely contained any detectable steroid material. When pregnancy urine was acidified to pH 1 and separate aliquots extracted after 0, 2, 4 and 24 hr., paper chromatography showed that generally there was a certain amount of destruction of steroids after 24 hr. at pH 1.

Heard, Sobel and Venning (1946) showed that if urine was acidified to pH 1 the amount of reducing substances that could be extracted was doubled but if the urine after acidification was neutralized/

neutralized before extraction an increased amount of reducing substances was not obtained. The method used by these workers measures those compounds which will reduce phosphomolybdic acid. This acid is not only reduced by primary α-ketol groups, such as are found in the side-chains of the adrenocortical hormones, but also by secondary α-ketol groups and αβ-unsaturated-3-ketosteroids and the reducing power of these groups is influenced by the presence of other functional groups in the steroid molecule. When a urine extract was allowed to stand in 2% sulphuric acid at room temperature for 1 hr. 60% of the reducing power was lost. When a solution of 11-deoxycorticosterone was treated similarly 25% of the reducing power was lost and oxidation of the extract with periodic acid showed that the α-ketol side-chain had not been affected. Thus it is probable that the loss of reducing power of urine extracts on acidification was due not to destruction of the side-chain but to transformation of some other part of the molecule if the material destroyed was steroidal.

While the presence in urine of FSS unstable at pH 1 at room temperature for 24 hr. seems well proved the presence of FSS labile in one hour has/

has not been shown by any of the above investigators. It is known that the formaldehydogenic properties of cortisol, cortisone, 11-deoxycorticosterone and 17-hydroxy-11-deoxycorticosterone are unaltered on standing in aqueous solution at pH 1 at room temperature for 24 hr. and the present author has shown by visual estimation after paper chromatography that the above steroids are quantitatively recovered after standing at pH 1 for one hour. Although these solutions of pure steroids are stable under the above conditions it does not necessarily follow that the steroids will be as stable in urine. It was thought early in the investigations that the cold acid-labile FSS might be aldosterone or one of its metabolites. However, it has been shown by Simpson, Tait, Wettstein, Neher, von Euw, Schindler and Reichstein (1954) that 80% of aldosterone is recovered after standing for twenty hours in aqueous solution at pH 1. Also the amounts of cold acid-labile FSS detected by Paterson and Marrian greatly exceed the amount of aldosterone detected in urine. The latest and largest value for the aldosterone content of urine is 3.5 µg. per 24 hr. Luetscher and Axelrad (1954) found the excretion of aldosterone to

be/

be increased above normal by restricting the sodium intake but the patient on a salt-free diet studied in the present work showed no definite increase in excretion of cold acid-labile FSS.

Determination of small amounts of cold acid-labile FSS are within the limits of experimental error of the method used. With the aliquots of urine normally used in the determinations a difference in Spekker readings of 0.005 between the 0 hr. and 1 hr. extracts would give a value for acid-labile FSS of 4 to 5.4 μg. HCHO per 24 hr. For this reason values of less than 6.0 μg. HCHO per 24 hr. have been regarded as insignificant. Duplicate determinations of FSS may vary by a Spekker reading of 0.004.

Paterson and Marrian found the values of cold acid-labile FSS to range from 8.4 to 27.4 μg. HCHO per 24 hr. Although it is possible to ascribe some of this to FSS in the solvents used it does not seem possible to account for all the labile FSS found by the above authors in this way. Nor can it explain the rise in labile FSS found by Paterson and Marrian after the injection of ACTH nor the few cases in the present work where/

where relatively large amounts (greater than 6 μg. HCHO per 24 hr.) of labile FSS were detected. It is certain that if cold acid-labile FSS do exist in urine they are present in much smaller quantities than suggested by the work of Paterson and Marrian. Taking the above facts into account it was thought that a continuation of the work at this time would not be profitable.

PART III. THE ATTEMPTED ISOLATION OF TETRA-HYDROCORTISONE GLUCURONOSIDE FROM NORMAL MALE URINE.

1. Introduction.

Tetrahydrocortisone ($3\alpha:17\alpha:21$-trihydroxy-5β-pregnane-11:20-dione) was first isolated from normal human urine by Schneider (1950c) who obtained 6 mg. as the diacetate from 1000 litres of unhydrolysed urine. It was subsequently isolated from normal urine by Baggett, Kinsella and Doisy (1953) and from the urine of patients treated with ACTH or cortisone by Lieberman, Katzenellenbogen, Schneider, Studer and Dobriner (1953). These two groups of workers and also Schneider (1952) obtained the compound after the urine had been hydrolysed with β-glucuronidase preparations and it appeared that most of the tetrahydrocortisone was present in a conjugated form, most probably as the glucuronoside. Determinations by Baggett <u>et al</u>. of the daily excretion of tetrahydrocortisone showed that this varied from 2.0 to 7.4 mg. so that the amount of tetrahydrocortisone glucuronoside present in urine would be about 3 to 11 mg. per day. Thus it was considered that the isolation of the conjugate from normal human urine should be a feasible proposition.

A procedure which it appeared might allow the/

the conjugate to be isolated was worked out but before it could be applied to a large scale attempt the isolation of tetrahydrocortisone glucuronoside from the urine of a man fed 2 g. of tetrahydrocortisone was reported by Schneider, Lewbart, Levitan and Lieberman (1955). In view of this the problem was not pursued further. This part of the thesis describes briefly procedures, not recorded in chronological order, that were tried in the attempt to isolate the conjugate.

2. Methods.

Extraction of urine.

Urine, and other aqueous solutions to be extracted with butanol unless otherwise stated, were acidified with dilute sulphuric acid to pH 1 (pH checked using a glass electrode pH meter), saturated with sodium chloride and extracted three times with 1/4 vol. of butanol. Any emulsions that formed were broken by centrifugation. The butanol extracts were washed with 1/10 vol. of water, neutralized with methanolic sodium hydroxide and the butanol removed _in vacuo_ using an air leak and a water bath temperature of about 60°C.

Estimation/

Estimation of tetrahydrocortisone glucuronoside.

Since methods of estimating the conjugate directly were unsatisfactory the rather laborious procedure of hydrolysing the conjugate with an enzyme preparation, extracting the free tetrahydrocortisone and estimating this after paper chromatography was adopted.

The residue of the fractions to be analysed was dissolved in water and an aliquot estimated to contain about 50 µg. of the conjugate was removed. The volume was made up to 8 ml. by the addition of water, the pH of the solution adjusted to 4.7 and 2 ml. of M acetate buffer pH 4.7 and 10 mg. of the soluble enzyme preparation described below added. The mixture was incubated at 37°C. overnight and the next morning extracted twice with 2 vol. of chloroform. The chloroform extract was washed twice with 1/5 vol. of 0.1 N-sodium hydroxide solution, twice with 1/5 vol. of distilled water, dried over anhydrous sodium sulphate and evaporated to dryness *in vacuo*. The residue obtained was dissolved in a small amount of methanol and applied to Whatman No.42 filter paper for chromatography using the solvent mixture toluene:ethyl acetate:methanol:water (85:15:60:40), a/

a modification of system C of Bush (1952). The papergrams were sprayed with alkaline blue tetrazolium solution and the amount of tetrahydrocortisone present estimated visually.

The R_F value of tetrahydrocortisone in this system was 0.20. A blue tetrazolium reducing spot with an R_F value of 0.60 was also present on the papergrams, the amount of this substance present being about half that of tetrahydrocortisone. This substance, if a steroid, was also present in a conjugated form and liberated by the enzyme preparation used. Whilst it behaved like tetrahydrocortisone glucuronoside in the extraction procedures it could be separated from the tetrahydrocortisone conjugate by the counter-current distribution and partition chromatography procedures described below. If this less polar substance is a steroid it may not have a hydroxyl group at position 17 since Dr Forsius (personal communication) who has been studying steroid conjugates in the urine of a person receiving cortisone has not detected a conjugate less polar than that of tetrahydrocortisone. Dr Forsius has used the method of Porter and Silber (1950) which/

which is claimed to be specific for those steroids with a dihydroxyacetone side-chain for estimating the conjugated metabolites of cortisone so that his inability to detect this compound less polar than tetrahydrocortisone may indicate that this compound does not have a hydroxyl group at position 17.

Preparation of concentrates of the enzyme β-glucuronidase.

Dodgson, Lewis and Spencer (1953) have shown that certain marine molluscs are a rich source of β-glucuronidase and the procedure devised by Dodgson and Spencer (1953) for the preparation of concentrates of this enzyme has been followed.

Limpets (Patella vulgata) were collected locally from the sea shore. They were removed from their shells and the visceral humps cut off. The pooled viscera (about 200 g.) were macerated in about 1 litre of acetone (previously chilled) for 30-60 sec. and then quickly filtered by suction. The debris was again macerated with 500 ml. of acetone, filtered and well washed with cooled/

cooled acetone. The debris was sucked free from liquid and dried *in vacuo*.to give a light grey powder.

This powder was then suspended in ten times its weight of water and the mixture well stirred. The suspension was centrifuged and the supernatants filtered through glass wool. The sedimented debris was resuspended in six times its weight of water and the process repeated. To the combined supernatants 8 vol. of cooled acetone were added to precipitate the protein. The main bulk of this supernatant was decanted, the remainder filtered and the residue, while still wet was well washed with acetone and finally dried *in vacuo*. The resulting water soluble white powder was used as the enzyme preparation.

Determination of the activity of the enzyme preparation.

The method described by Fishman, Springer and Brunetti.(1948) has been used. A solution of the enzyme was made by dissolving 2 mg. of the powder in 10 ml. of water. The enzyme solution (0.1 ml.), acetate buffer 0.1 M, pH 4.7 (0.8 ml.) and 0.01 M solution of phenolphthalein glucuronide adjusted/

adjusted to pH 4.7 (0.1 ml.) were mixed and incubated for 1 hr. in a water bath at 37°C. The reaction was stopped by the addition of 1 ml. of 5% trichloracetic acid and 2.5 ml. of buffer (pH 10.4 prepared by dissolving 32.6 g. of glycine, 25.3 g. of sodium chloride and 15.5 g. of sodium hydroxide in 2 litres of water) and 1.5 ml. of water added. The resulting solution was read at wavelength 540 mμ against a reagent blank and the amount of phenolphthalein released calculated from a calibration curve. Usually 1 g. of the water soluble powder had an activity of about 1,500,000 units. (1 unit is defined as the μg. of phenolphthalein liberated in 1 hr. by 1 g. of the enzyme preparation).

3. Purification of the conjugate by extraction procedures.

It was decided to try first some of the extraction procedures that had proved successful in the isolation of oestriol glucuronoside from urine (Cohen and Marrian, 1936; Cohen, Marrian and Odell, 1936).

Extraction/

Extraction of the conjugate from urine by butanol.

A sample of urine (150 ml.) was divided into three equal parts. One was hydrolysed enzymically, the second extracted with butanol at neutral pH and the third extracted after acidification to pH 1 with sulphuric acid. The residues obtained from the butanol extracts were dissolved in 50 ml. of water and hydrolysed enzymically. The results showed that the conjugate of tetrahydrocortisone, like that of oestriol, was extracted by butanol from urine acidified to pH 1 but not from unacidified urine.

Extraction of the conjugate from butanol by aqueous alkali.

A butanol extract of urine was divided into two equal parts. One part was evaporated to dryness and the residue hydrolysed by enzyme. The other half was neutralized and extracted twice with 1/2 vol. of 5% (w/v) sodium bicarbonate solution. The butanol was next extracted twice with 1/2 vol. 0.3 N-sodium hydroxide solution. These alkali extracts were neutralized and hydrolysed by enzyme. Finally the butanol remaining from the alkali extractions was evaporated/

evaporated to dryness and the residue hydrolysed. The results showed that the bicarbonate had extracted about 75% of the conjugate. The sodium hydroxide had extracted the remaining 25% and none was left in the butanol. Oestriol glucuronoside is also extracted from butanol by 0.8% sodium carbonate solution and by sodium hydroxide solution (Cohen and Marrian, 1936; Cohen et al., 1936).

In another experiment it was shown that by acidification of the alkali extracts the conjugate could be extracted with butanol and from the amount of residue obtained by evaporation of the butanol it was obvious that a high degree of purification had been obtained. Only a small amount (about 15%) of the conjugate was extracted by butanol from the alkaline phase before acidification.

Distribution between quinoline and aqueous alkalis.

Cohen and Marrian (1936) had found that oestriol glucuronoside could be extracted from acidified aqueous solutions by quinoline. Washing the quinoline solution with sodium carbonate solution removed much pigmented material but only traces of the conjugate. Most of the conjugate could/

could be recovered by extracting with sodium hydroxide. A similar procedure was tried with the conjugate of tetrahydrocortisone.

An aliquot of an aqueous solution of the residue from a butanol extract of urine was hydrolysed enzymically. The remainder of the solution was acidified to pH 1 and extracted twice with 1/2 vol. of quinoline. The quinoline extract was then successively extracted twice with 2 vol. of 5% sodium bicarbonate solution, twice with 2 vol. of 10% sodium carbonate solution and twice with 2 vol. of 0.1 N-sodium hydroxide solution. Aliquots of the alkali solutions and of the quinoline extracted aqueous phase were then hydrolysed. The results showed that while quinoline effectively removed the conjugate from an acidified aqueous solution, sodium bicarbonate would extract only about 50% of the conjugate from the quinoline phase and this bicarbonate extract was highly pigmented. A further amount of the conjugate (10-25%) was recovered in the sodium hydroxide fraction.

Extraction of an aqueous solution of the conjugate at pH 7.4 with quinoline removed only a small amount of pigment and none of the conjugate. Thus/

Thus the quinoline procedure was of no use in the purification of tetrahydrocortisone glucuronoside.

Extraction of tetrahydrocortisone glucuronoside from aqueous solutions by ether, chloroform and ethyl acetate.

Lieberman and Dobriner (1948) have reported that when sodium pregnanediol glucuronosidate is dissolved in aqueous acid solution (pH 1) and continuously extracted with ether the unhydrolysed conjugate is obtained in the ether extract. When an aqueous acid (pH 1) solution of a residue obtained from a butanol extract of urine was continuously extracted with ether for seven hours it was shown that none of the tetrahydrocortisone glucuronoside had entered the ether phase and the conjugate was recovered from the aqueous phase.

It was also shown that the conjugate could not be extracted from aqueous solution by chloroform.

When an aqueous solution of the conjugate was acidified to pH 1 and extracted twice with 1 vol. of ethyl acetate the conjugate was approximately equally distributed between the two phases. None of the conjugate and little pigment were extracted by ethyl acetate from an aqueous solution at neutral pH.

Butanol has been the most widely used solvent for the extraction of steroid conjugates from urine. The effect of pH on the distribution of the conjugated 17-ketosteroids between urine and butanol was studied by Bitman and Cohen (1951). At a pH less than 3 all the conjugated 17-ketosteroids were extracted but at higher pH values considerable amounts of the conjugates were not extracted. A similar finding has been reported by Crepy, Garrone and Jayle (1952) and these workers have used the method of fractionating the conjugates by extracting urine at different pH values with butanol to determine the excretion of different groups of steroids. Anderson and Warren (1951) have studied the extraction of conjugated androgens from acidified urine by different solvents. Using 1/20 vol. of each solvent they found that _n_-butanol extracted 88% of the conjugate, _iso_- and _sec_-butanol 58% and _tert_-amyl alcohol 65%. Ether, chloroform and ethylene dichloride removed 11, 8 and 5% respectively, while toluene removed a negligible amount. Brooksbank and Haslewood (1950) found that 3α-hydroxy-5α-androst-16-ene glucuronoside was appreciably soluble in chloroform.

Edwards/

Edwards, Kellie and Wade (1953) have shown that steroid conjugates can be quantitatively extracted from urine to which ammonium sulphate has been added by ether-ethanol mixtures. Butanol was equally effective while ethyl acetate removed 80% of the 17-ketosteroid conjugates and ether only 24%. Fishman and Sie (1956) have used ethyl acetate to extract testosterone glucuronoside, synthesized <u>in vitro</u> by liver slices of various animals, from an acidified aqueous solution. Thus while butanol or ether-ethanol mixtures appear to be the best solvents for extracting the conjugates from urine purification of the conjugates could perhaps be achieved by using extraction with various solvents at later stages in the purification procedure.

4. Purification of the conjugate by counter-current distribution.

The theory of counter-current distribution has been reviewed by Craig and Craig (1950). In the work described below separating funnels have been employed for they allow large volumes of each phase to be used. Due to the laboriousness of the/

the procedure only a small number of transfers have been applied to the mixture containing the conjugate with the intention of removing the main bulk of impurities present. It was thought that this could best be achieved by finding a solvent system in which the conjugate had a partition coefficient of 1.0 so that the peak of the concentration of the conjugate would be at the centre of the distribution and the impurities present mainly at each end.

Distribution using the solvent mixture butanol: 0.5 M acetate buffer pH 4.7.

Bitman and Cohen (1951) have reported that steroid glucuronosides have a pK value between 3 and 4.5. Thus it was expected that the conjugate of tetrahydrocortisone would be equally distributed between butanol and an aqueous solution of about pH 4.5. When an extract of urine containing the conjugate was subjected to a 5 funnel distribution using the above system it was found that most of the conjugate was in funnel 3 with smaller amounts in funnels 2 and 4. Funnels 1 and 5 although they contained little conjugate contained much pigment and other material so that a considerable degree of/

of purification of the conjugate had been achieved.

The volume of each phase used in the distribution varied with the amount of material to be distributed and the procedures that had already been applied in its purification. Phase volumes of 100 ml. to 1 litre have been used. At the end of the distribution the aqueous phases were run off, adjusted to pH 1, extracted with the butanol phase and then extracted again with 1/2 vol. of butanol.

Although much purification was achieved by use of this system it had the disadvantage that the butanol tended to remove acetic acid from the lower phase so that there was a gradual change in pH as the distribution proceeded.

Distribution using the solvent mixture ethyl acetate:dilute sulphuric acid pH 1.5.

It was found that tetrahydrocortisone glucuronoside also had a partition coefficient of about 1.0 in the above system. At the end of the distribution the ethyl acetate was removed and evaporated to dryness and the aqueous phase in each funnel extracted twice with 1/2 vol. of butanol. The residue obtained by evaporation of the/

the butanol from each funnel was combined with the corresponding residue obtained from evaporation of the ethyl acetate. It was shown in another experiment that no loss of conjugate occurred during evaporation of the ethyl acetate.

This system appeared to have a lower capacity than the butanol:acetate buffer system but was very useful in the purification of concentrates of the conjugate.

Counter-current distribution has been used by Schneider et al. (1955) for the isolation of tetrahydrocortisone conjugate from urine; the solvent mixture ethyl acetate:butanol:0.01 N-hydrochloric acid was used. More recently Lewbart and Schneider (1956) have employed counter-current distribution using the solvent mixture ethyl acetate:n-hexane:1% acetic acid to isolate androsterone glucuronoside from urine. The only other reported application of counter-current distribution for the separation of steroid conjugates is that of Saier, Grauer and Strickler (1956). Using fifty transfers no separation of the sodium 3α:20α-dihydroxy-5β-pregnane glucuronosidate/

glucuronosidate complex was obtained with the system butanol:0.1 N-sodium hydroxide solution.

5. Purification of the conjugate by adsorption chromatography.

The theory of adsorption chromatography has been reviewed by Cassidy (1951). It was anticipated that the most commonly used adsorbent, alumina, would be of little use since it is known that the free corticoids are strongly adsorbed and tend to be destroyed. Brooksbank and Haslewood (1950) found that after chromatography of sodium pregnanediol glucuronosidate on an alumina column only 20% was recovered. The recovery of their 'pregnanediol-like glucuronosidate' after chromatography was about 80% but no clear cut separation was achieved. Alumina has also been used by West, Reich and Samuels (1951), again with little success. Ethanol-acetic acid mixtures were needed to elute the conjugates and a mixture of the conjugates of androsterone and etiocholanolone was not resolved.

The following adsorbents were tested:-

Deactivated/

Deactivated magnesium silicate-Celite.

A 1:1 mixture of magnesium silicate, deactivated by exposure to the atmosphere, and Celite was used. The partially purified extract of urine was dissolved in 1 ml. of ethyl acetate and applied to the column. The conjugate was not eluted by ethyl acetate or ethanol.

Celite.

A slurry of Celite in ethyl acetate was made and poured into a column. The column was packed under pressure and the partially purified extract of urine dissolved in 1 ml. of ethyl acetate and applied to the column. The conjugate appeared in the first 10 ml. of eluate.

Starch.

This was dried by heating overnight at 80°C. and a column prepared in ethyl acetate. The conjugate appeared in the first 20 ml. of eluate so that little or no adsorption had taken place.

None of the three adsorbents tested was of use in the purification of the conjugate for the conjugate/

conjugate was either so strongly adsorbed that it could not be eluted or, if adsorbed, the adsorption was so weak that the conjugate was eluted with the main band of pigment and impurities. A separation of the methylated and acetylated glucuronosides of 17-oxosteroids on alumina has been reported by Kellie and Wade (1956).

6. Purification of the conjugate by partition chromatography.

It was considered that partition chromatography would be more suitable than adsorption chromatography since, due to the milder conditions, there would be less chance of destruction taking place and the resolution obtained is greater. The equation derived by Martin and Synge (1941) has been modified by Butt, Morris, Morris and Williams (1951) and expressed as:-

$$K = \frac{Vr}{LAs} - \frac{A_L}{A_s}$$

where K = the partition coefficient $\frac{\text{conc. in stationary phase}}{\text{conc. in mobile phase}}$

Vr = retention volume i.e. vol. of eluate to peak conc. of solute

L = length of column

A_L = cross-sectional area of mobile phase and

A_s = " " stationary phase

By/

By rearranging this equation:-

$$Vr = K\ L\ A_s + LA_L$$

and thus under ideal conditions and for similar columns the retention volume of a solute depends only on its partition coefficient. Since for the best resolution the solute should not leave the column too early it is obvious that the solute should favour the stationary phase i.e. increasing K increases Vr. In practice values of K from about 10 to 20 are best for if K is too large the resolution tends to become blurred due to diffusion taking place between the 'plates' of the column.

The following systems have been investigated:-

0.1 N-Sodium hydroxide solution:butanol.

Whilst tetrahydrocortisone glucuronoside was readily extracted with butanol from acidified urine little was extracted at neutral pH. The preference of the salt form of the conjugate for the aqueous phase suggested that this solvent system would probably be suitable for chromatography of the conjugate.

Celite/

Celite was used as supporting medium and the method of packing the columns has been described by Bauld (1955). Eight ml. of sodium hydroxide were used per 10 g. of Celite and the extract containing the conjugate was applied to the column by dissolving it in a small volume of stationary phase, stirring in the requisite amount of Celite and packing this Celite onto the top of the column. The fractions of the eluate were neutralized and evaporated to dryness *in vacuo*. Using this system the conjugate of tetrahydrocortisone usually appeared in the third and fourth fractions.

However this solvent system had certain disadvantages. One was that stationary phase tended to be lost from the column with the result that the conjugate appeared in the eluate more quickly than was desired. No experiments were performed in which the proportion of stationary phase to Celite was varied. The amount used should not have been excessive and the slurry obtained on adding mobile phase to the Celite was the flocculent type recommended by Bauld. Dr Forsius (personal communication) has also found this solvent system to behave in a similar manner.

Secondly/

Secondly it is known that steroids with an α-ketol group are unstable in alkaline solution so that the eluate had to be neutralized as soon as it was collected.

Citrate-phosphate buffer pH 2.5:ethyl acetate.

It was known that ethyl acetate would not extract the conjugate from a neutral aqueous solution although it would from an acidified (pH 1) solution. Thus it should be possible to find a buffer solution of suitable pH value which could be used for the stationary phase. A citrate-phosphate buffer (McIlvaine, 1921) was found to be the most suitable and a pH of 2.5 to 2.7 appeared to be the optimum value. Employing this system the first 20 ml. of eluate were always highly pigmented. The next 20 ml. of eluate were almost free of pigment and contained the conjugate. This system was most successful and the retention volume of the conjugate could be varied by varying the pH of the buffer.

Water:butanol.

For this solvent system starch was used as the support. The starch was made into a slurry with water-saturated butanol and poured into the column/

column. Since the flow rate was extremely slow in later experiments a mixture of equal amounts of starch and Celite was used. The system was unsatisfactory for contact between the water-saturated butanol and starch caused a decrease in pH of the system with the result that the conjugate was eluted together with the pigment in the extracts and appeared in the first fraction of the eluate. Using a mixture of starch and sodium bicarbonate as the supporting medium or using a phosphate buffer of pH 7 as stationary phase instead of water did not lead to any improvement. Stein and Moore (1948) have studied the characteristics of starch columns and have shown that the starch needs to be treated with 8-hydroxyquinoline to remove traces of metal ions. The source of the starch was most important and potato starch was by far the most satisfactory. Other disadvantages were that the columns had a low capacity and that the eluate was contaminated with traces of carbohydrate.

Sutherland and Marrian (1947) using a column of potato starch in moist butanol obtained no separation of the 'sodium pregnanediol glucuronosidate/

glucuronosidate' mixture into ketonic and non-ketonic fractions and West, Reich and Samuels (1951) used a similar column for the separation of the conjugates of testosterone metabolites but with no success. Edwards and Kellie (1956) have successfully separated the 17-ketosteroid conjugates present in the urine of a patient injected with testosterone by chromatography of the ketonic fraction of the urine extract on silica gel using 0.1 N-sodium acetate solution as stationary phase and chloroform containing 2% ethanol and varying amounts of acetic acid as the mobile phase. Enzymic hydrolysis of the major peaks from the column showed the presence of androsterone, etiocholanolone and their 11β-hydroxy derivatives and also testosterone. This is the first demonstration *in vivo* of the conjugation of a C-19 steroid in a position other than C-3. Fishman and Sie (1956) have shown *in vitro* that testosterone is conjugated with glucuronic acid by liver slices from various animals. It should be pointed out that silica gel columns are not true partition columns for a considerable degree of adsorption takes place. This/

This may not always be advantageous. The only other report on the application of partition chromatography to the separation of steroid conjugates is that of Anderson and Warren (1951). These authors tried reversed phase chromatography using butanol as stationary phase supported on Celite treated with dichlorodimethylsilane. The column used extracted 94% of the conjugates from acidified urine.

When the work described in this part of the thesis was commenced no report on the successful application of partition chromatography to the separation of steroid conjugates had appeared.

7. Discussion.

Few steroid glucuronosides have so far been isolated from urine. Of those that have only four have been obtained in a pure or nearly pure state. The first to be isolated was oestriol glucuronoside (Cohen and Marrian, 1936; Cohen *et al*., 1936). A butanol extract of pregnancy urine was extracted with sodium hydroxide solution. After acidification this solution was re-extracted with butanol and the residue from evaporation of the butanol subjected to the quinoline procedure. The/

The purified oestriol glucuronoside was crystallized as the sodium salt from methanol. Venning and Browne (1936) isolated a glucuronoside of 5β-pregnane-3α:20α-diol also from pregnancy urine. The residue from a butanol extract of urine was dissolved in alkali and re-extracted with butanol. The residue from this butanol extract was dissolved in water and the conjugate precipitated by the addition of acetone. In 1946 Marrian and Gough showed that 'sodium pregnanediol glucuronosidate' prepared by the method of Venning and Browne contained about 20% of a conjugate of 3α-hydroxy-5β-pregnan-20-one and Sutherland and Marrian (1947), by subjecting the conjugate mixture to a modified Girard separation, were able to isolate pregnanediol glucuronoside in an almost pure state. From the ketonic fraction they obtained the glucuronoside of 3α-hydroxy-5β-pregnan-20-one which was about 85% pure.

No other steroid glucuronoside was isolated in a pure state until 1955 when Schneider _et al_. isolated the glucuronoside of tetrahydrocortisone from the urine of a man who had received 2 g. of tetrahydrocortisone over a period of four hours. The/

The urine was collected for 16 hr., extracted at pH 1 with butanol and evaporation of the butanol left a residue which contained 50% of the steroid conjugate. This residue was distributed in a counter-current machine using the system ethyl acetate:butanol:0.01 N-hydrochloric acid (9:1:10) and pure tetrahydrocortisone glucuronoside was obtained although it was not crystallized. The solvent mixture used is similar to one (ethyl acetate:sulphuric acid pH 1.5) used in the present work.

Recently Lewbart and Schneider (1956) have isolated androsterone glucuronoside from the urine of a man treated with dehydro*epi*androsterone. The residue from a butanol extract of the urine was dissolved in 1% aqueous acetic acid and this solution was repeatedly extracted with ethyl acetate. The residue obtained from evaporation of the ethyl acetate was distributed in a counter-current apparatus using the solvent mixture ethyl acetate:*n*-hexane:1% aqueous acetic acid (6:3:9) and crystalline androsterone glucuronoside was obtained.

A number of steroid glucuronosides have been isolated in a semi-pure state. The conjugate of

of 3α:17α-dihydroxy-5β-pregnan-20-one was isolated by the method of Venning and Browne from the urine of a female pseudohermaphrodite (Strickler, Shaffer, Wilson and Strickler, 1943; Mason and Strickler, 1947). Brooksbank and Haslewood (1950) isolated 3α-hydroxy-5β-androst-16-ene glucuronoside by extracting normal male urine with butanol at neutral pH, entrainment of the residue from the butanol on barium phosphate and chromatography on alumina. The final product contained 5β-pregnane-3α:20α-diol in addition to other unidentified material. By treating the urine from a female pseudohermaphrodite according to the Venning and Brown method Mason and Kepler (1945b) isolated a crystalline fraction whose melting point was not raised by repeated crystallization. However hydrolysis showed that this crystalline material was a mixture of conjugates and from the hydrolysate 5β-pregnane-3α:20α-diol, 3α:17α:20α-trihydroxy-5β-pregnane and at least four ketonic compounds were isolated. West, Reich and Samuels (1951), again using the Venning and Brown method with the urine from a man receiving testosterone, obtained/

obtained a product which behaved as a single substance on chromatography on paper and alumina but which yielded a mixture of androsterone and etiocholanolone on enzymic hydrolysis. Tetrahydrocortisone glucuronoside was not precipitated from aqueous solution by the addition of acetone.

Thus it is seen that the first isolation of a conjugate in every case except one has been from urine in which there was an amount of the conjugate greatly in excess of that found in normal urine. It has been shown that many other steroids are probably also excreted as glucuronosides (Lieberman and Dobriner, 1948; Romanoff, Wolf, Constandse and Pincus, 1953). Only in the case of the oestriol conjugate has it been shown by isolation that the acid is glucuronic acid (Grant and Marrian, 1950) but in the case of the conjugates of pregnanediol and tetrahydrocortisone the isolated conjugate or a derivative has been shown to be identical with the same compound synthesized from the steroid and glucuronic acid.

A number of solvent mixtures suitable for the separation of steroid sulphates and glucuronosides by paper chromatography have recently been described by Lewbart and Schneider (1955) but have not yet been applied to urine extracts. Previously while West/

West *et al*. (1951) had separated the sulphates of androsterone and dehydro*epi*androsterone by paper chromatography in the butanol:water system a separation of the glucuronosides of androsterone and etiocholanolone was not achieved.

The discovery of methods for purifying and isolating the conjugates is important for devising methods for the quantitative estimation of steroids, especially those originating in the adrenal cortex, in urine. It is known that many steroids are transformed during hydrolysis with acid and enzymic hydrolysis is also unsatisfactory for a method which is to be used routinely. Not only does the enzymic hydrolysis greatly lengthen the time required to perform an estimation but when applied to urine hydrolysis is possibly not complete and variable since competitive inhibition may occur due to the presence of non-steroid glucuronosides in urine and in addition urine may also contain other substances that inhibit the activity of the enzyme. Some of the difficulties encountered in quantitatively determining urinary corticosteroids have been discussed by Marrian (1951). It would appear that the best method of estimating urinary steroids would be to estimate the conjugates so that hydrolysis procedures are avoided. Although the/

the extraction of the conjugates may not be quantitative it is known that the extraction of the highly oxygenated corticosteroids from urine by the usual extraction procedures is not quantitative either (Baggett _et al._, 1953; Fukushima _et al._, 1955; Nadel _et al._, 1956). In addition to the usual extraction procedures it should be possible to separate the conjugates by use of a counter-current distribution apparatus of the type described by Craig, Hausmann, Ahrens and Harfenist (1951) although this is unlikely to become a routine procedure. Another possible method would be the use of ion-exchange resins. Only one report has appeared concerning the use of these resins for the concentration of urinary conjugates (Anderson and Warren, 1951) and in this case the trials were unsuccessful.

Thus although the primary aim when the work described here was commenced has not been attained much has been learnt of the properties of tetrahydrocortisone glucuronoside, the most abundant urinary corticosteroid conjugate, and this information may be of use in the elaboration of methods for the estimation of urinary corticosteroids.

APPENDIX I. THE ISOLATION OF FURTHER CRYSTALLINE COMPOUNDS FROM EXTRACTS OF NORMAL MALE URINE.

As can be seen from Table 14 (Appendix 2) only two steroids containing the 5-en-3β-ol group have been isolated from the urine of normal people. The residues of the urine extracts from which K2 had been isolated had been stored and therefore provided ideal starting material for investigating the presence in normal urine of compounds containing the 5-en-3β-ol group.

The urine residues were subjected to the Girard separation outlined in Fig. 3 (Part I) with the modification that ether instead of chloroform was used to extract the non-ketonic and ketonic fractions so that caffeine which appears in the non-ketonic fraction when chloroform is used would not be extracted. The weights of the fractions were:-

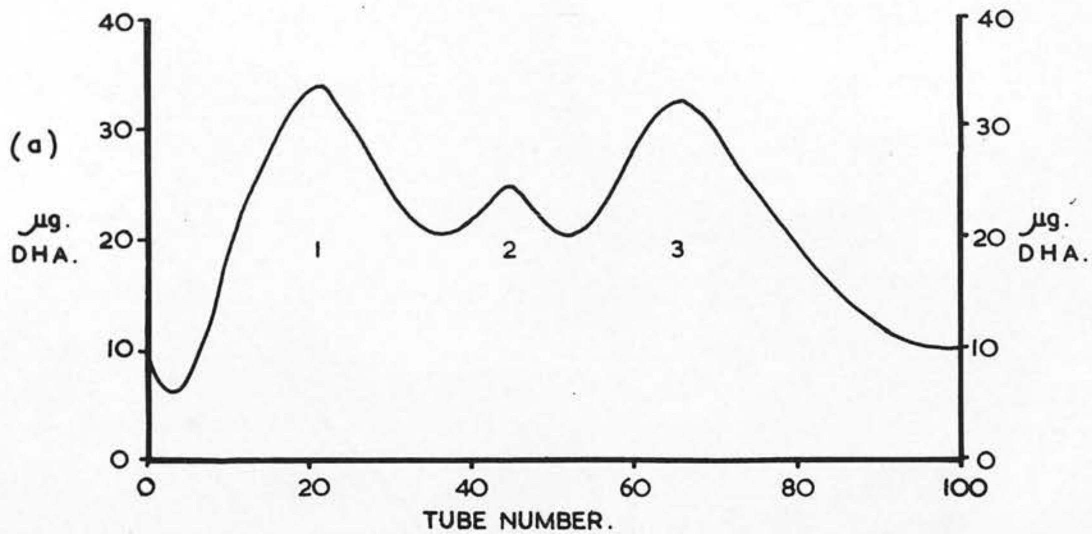
Non-ketonic 1.5125 g.
Ketonic 0.9133 g.

These fractions were then distributed in a 100 tube all-glass counter-current apparatus of the type described by Craig _et al_. (1951). The partition coefficients for a few neutral steroids in various solvent mixtures composed of ethyl acetate, _cyclo_hexane, ethanol and water have been determined by Engel, Alexander, Carter, Elliott and/

and Webster (1954) and the above solvent mixture has been used in the present study. Unfortunately the partition coefficient of only one steroid with a 5-en-3β-ol group, namely dehydro*epi*androsterone, in this system was known. The two phases of the system were mutually saturated before use and the distributions were performed in a constant temperature room maintained at 25°C. An upper and lower phase volume of 40 ml. per tube has been used in both experiments.

Distribution of the non-ketonic fraction.

For this distribution the solvent mixture ethanol:water:*cyclo*hexane: ethyl acetate 8:2:8:2 was employed. In this system the partition coefficient of dehydro*epi*androsterone is 0.21. The non-ketonic fraction was dissolved in 40 ml. of lower phase and placed in tube 0 of the machine. After completion of the fundamental procedure the upper phase was recycled for 300 cycles. The distribution pattern was then determined by removing 0.2 ml. of each phase from each tube and subjecting these to the Pettenkofer reaction described in Part I. The distribution pattern is shown in Fig. 17a. Three peaks were clearly seen and/



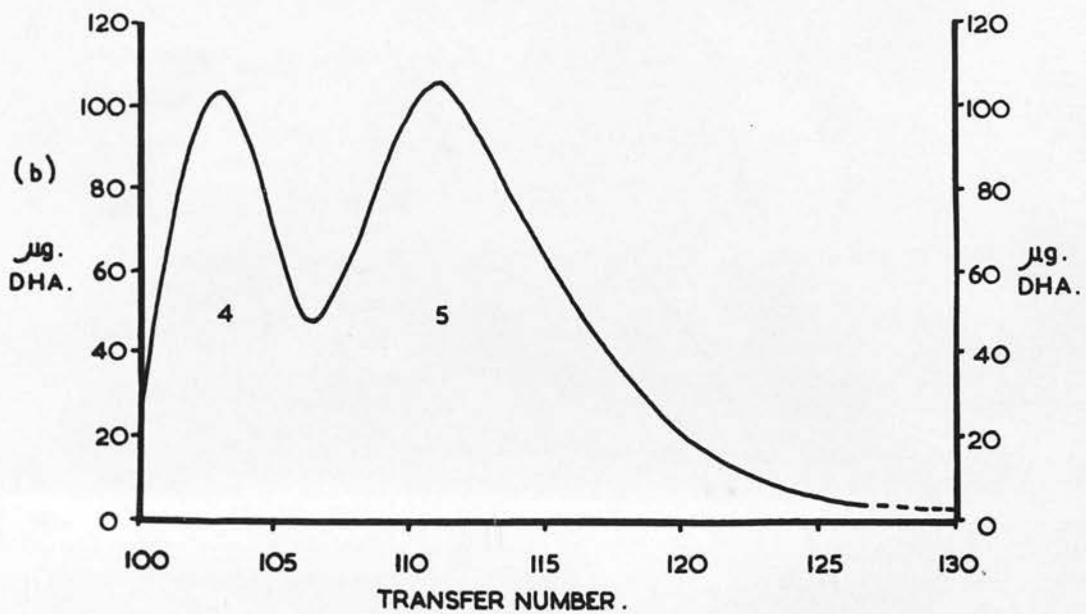


FIG.17. DISTRIBUTION PATTERN FOR EXTRACTS OF URINE.
(a) NON-KETONIC FRACTION. (b) KETONIC FRACTION.

and the contents of the tubes shown below were combined, the solvents removed in vacuo and the residues weighed.

Peak 1	Tubes 9-38	Weight 572 mg.
Peak 2	Tubes 39-52	Weight 232 mg.
Peak 3	Tubes 53-80	Weight 369 mg.

Distribution of the ketonic fraction.

For this distribution the solvent mixture ethanol:water:cyclohexane:ethyl acetate 3:7:8:2 was employed. In this system the partition coefficient of dehydroepiandrosterone is approximately 12; it is not possible to arrive at a more accurate value from the nomograms of Engel et al. (1954). The ketonic fraction was dissolved in 40 ml. of the lower phase and placed in tube 0 of the machine. After completion of the fundamental procedure 100 fractions were collected by the single withdrawal method. These fractions were evaporated to dryness in a stream of air, the residues dissolved in 2 ml. of methanol and 1/200 of each alternate fraction used for the Pettenkofer reaction. The Pettenkofer reaction was also performed on 0.2 ml. of each phase from alternate tubes of the counter-current apparatus. The/

The distribution pattern is shown in Fig. 17b. Only two peaks were found one of which (number 4) moved almost with the solvent front.

Identification of the material in peak 3 of the non-ketonic fraction.

Due to the large amount of pigment present this fraction could not be induced to crystallize. It was therefore chromatographed on a 30 x 2 cm. column of Celite using 70% aqueous methanol (0.5 ml. per g. of Celite) as stationary phase and petroleum ether (boiling range 80-100°C.) as mobile phase. Little material was eluted by the petroleum ether so the column was washed with methanol and the residue obtained from the methanol washings rechromatographed on Celite using the solvent mixture benzene:70% aqueous methanol. Thirty three fractions each of 25 ml. were collected and the column was then washed with methanol. Crystalline material was obtained from fractions 5 to 11 and from fractions 23 to 29 but these have not yet been investigated further. Washing the column with methanol eluted a large amount of crystalline material. This was filtered in methanol and evaporation of the methanol left a/

a white residue weighing 53 mg. This residue was sparingly soluble in methanol and behaved in a similar manner to androst-5-ene-3β:16α:17β-triol. The material was crystallized twice from methanol to give 15 mg. of slightly pigmented crystals which melted from 250°C. to 257°C. Since the crystals were still obviously impure they were acetylated and the increase in weight on acetylation was 5.5 mg. Thus the

$$\% \frac{\text{Increase in wt. on acetylation}}{\text{Wt. of unacetylated compound}} = \frac{5.5 \times 100}{15}$$

$$= 36.7\%$$

Assuming the material to be androst-5-ene-3β:16α:17β-triol whose molecular weight is 306

$$\% \frac{\text{Increase in wt. on acetylation}}{\text{Wt. of unacetylated compound}} = \frac{126}{306}$$

$$= 41.2\%$$

The triacetate was crystallized once from methanol and once from 95% ethanol and the triacetate melted from 180 to 183°C. The melting point was not depressed when the triacetate was mixed with authentic 3β:16α:17β-triacetoxyandrost-5-ene. The presence of a 5-en-3β-ol group was indicated by the positive Pettenkofer reaction. It seems certain therefore that one component/

component of peak 3 was androst-5-ene-3β:16α:17β-triol.

This isolation of androst-5-ene-3β:16α:17β-triol has already been commented upon in the Discussion of Part I of this thesis. Since there is no evidence that the compound is present in the free state in urine it seems reasonable to conclude from the isolation above that it is present in urine as the 3β-sulphate. In a case of adrenal cortical tumour where the excretion of androst-5-ene-3β:16α:17β-triol was greatly increased Hirschmann (1943) could not extract any of the compound from urine with ether prior to acid hydrolysis.

The material in peak 2 of the non-ketonic fraction has not yet been investigated. When the material from peak 1 of the non-ketonic fraction was chromatographed on Celite using the solvent mixture benzene:80% aqueous methanol crystalline material was found in the first three fractions of eluate. These fractions were deeply pigmented and have not yet been investigated further. Washing the column with methanol eluted a large

amount/

amount of crystalline material which was deeply pigmented. This material was leached twice with 0.5 ml. of methanol to remove the pigment and the white powder obtained (wt. 55 mg.) was crystallized once from methanol and once from 80% aqueous ethanol. The melting point of the crystals obtained was 258-260°C. The nature of this compound is not yet known. From its chromatographic behaviour and its appearance in the non-ketonic fraction it is possible that it contains three hydroxyl groups.

Identification of the material in peak 5 of the ketonic fraction.

The residues contained in the tubes corresponding to transfer numbers 110 to 124 inclusive were combined and leached with 0.5 ml. of methanol. This leaching removed most of the pigment leaving a mass of pale yellow crystals. These were dissolved in methanol, the solution filtered and the material obtained from evaporation of the methanol crystallized from methanol at room temperature. The melting point of the white needles obtained was recorded:-

136°C. Melted
138° Partial resolidification
144° Beginning to melt again
148° Melt complete

It/

It was expected from its position in the distribution and from the large amount of it present that this material would be dehydro*epi*-androsterone. From the equation of Craig and Craig (1950):-

$$N = \frac{nK}{K+1}$$

where N = tube containing the maximum amount of the solute
n = number of transfers employed in the distribution
and K = the partition coefficient

we can calculate the position of the peak concentration of dehydro*epi*androsterone. The partition coefficient for dehydro*epi*androsterone in the solvent mixture used was approximately 12 and n = 199 so that N = 185. Thus the peak concentration of dehydro*epi*androsterone should appear in the tube corresponding to transfer number 115. From Fig. 17b it is seen that the peak concentration occurred in the tube corresponding to transfer number 113. The positive Pettenkofer reaction suggested that the compound possessed the 5-en-3β-ol group. The melting point of the isolated material was not depressed by admixture with pure dehydro*epi*androsterone. Thus/

Thus there can be little doubt that the material isolated was dehydro*epi*androsterone.

The material in peak 4 of the ketonic fraction has not yet been investigated. It is likely however to be a mixture of substances. One component will probably be K3 which has previously been isolated in small amounts in Part I of this thesis and which could be the 3β-chloro-derivative of K2. It is probable also that this peak will contain 3β-chloro-androst-5-en-17-one.

APPENDIX II. Tables 1, 2 and 14.

Table 1. C-19 steroids isolated from human urine. For abbreviations see Table 2.

Steroid	Melting point	$[\alpha]_D$	Melting point acetate	$[\alpha]_D$ acetate	H_2SO_4 Chromogen
Androst-3:5-dien-17-one	78-84	-31 E	-	-	
5α-androst-2-en-17-one	109-111	+148 E	-	-	
5α-androst-16-en-3α-ol	144-145.5	+20.9 C	132-132.5	+20.5 E	
3β-chloro-androst-5-en-17-one	156-158	+15.5 C	-	-	
3β-chloro-androst-5-en-17β-ol T	165-168		185-187		
5α-androst-1-ene-3:17-dione	138-142	+122 C	-	-	299 230
Androst-4-ene-3:17-dione T.H	170-171.5	+196 E	-	-	294 235
5α-androstane-3:17-dione	132-134	+112 E	-	-	302 250
5β-androstane-3:17-dione	130-131	+112 E	-	-	302 250
3β-hydroxy-androst-5-en-17-one (Dehydro*epi*androsterone)	139,147-149	+12 E	168-169	+4 E	304,404,482 250,344,456
6β-hydroxy-3:5-cycloandrostan-17-one (i-androstanolone)	140-141	+122 E	109-111	+117 E	
3α-hydroxy-5α-androst-9(11)-en-17-one	187-187.5	+136 E			

Table 1 contd.

Steroid	Melting point	[α]D	Melting point acetate	[α]D acetate	H_2SO_4 Chromogen
3α-hydroxy-5β-androst-9(11)-en-17-one	169-170	+151 A			
3β:17β-dihydroxy-androst-5-ene T	176-178	-56 E	158-160	-54 E	240,307,404,482 233,255,348,472 440,540
5β-androstane-3α:17β-diol	233-235	+26	125-127		343 250
3α-hydroxy-5α-androstan-17-one (Androsterone)	184-185	+97.7 E	160-162	+98.4 E	310,402,480 258,385,455 331
3α-hydroxy-5β-androstan-17-one (Etiocholanolone)	143-144, 150	+111 E	92-93.5		311,409,500 259,389,452 332
3β-hydroxy-5α-androstan-17-one (*epi*Androsterone)	175-176	+82 M	113-115	+69 C	310,407,490 252,389,450 331
3β-hydroxy-5β-androstan-17-one	154-155	+89 C	155-158		
3β-hydroxy-androst-5-en-7:17-dione H.T	245-248	-66.4 M	184.5-187.5	-72 E	

Table 1. contd.

Steroid	Melting point	$[\alpha]_D$	Melting point acetate	$[\alpha]_D$ acetate	H_2SO_4 Chromogen
11β-hydroxy-androst-4-ene-3:17-dione	190-195		-	-	283,380,460 241,322,433 402
3α-hydroxy-5α-androstane-11:17-dione	153.5-155	+127 E	183.5-184.5	+113 E	
3α-hydroxy-5β-androstane-11:17-dione	188-189	+95.8 E	163-164	+145.4 E	321,401 265,353 238,380,412
3α:11β-dihydroxy-5α-androstan-17-one H	199-200	+98.4 E	240-242	+90.4 A	
3α:11β-dihydroxy-5β-androstan-17-one H.T	237-238	+104 D	238		341,402 266,371 230,332
3α:17β-dihydroxy-5β-androstan-11-one	262-262.5	+68.4 E	182-183.5	+46.9 C	
3β:16α:17β-trihydroxy-androst-5-ene	264-266	-88 D	184-185	-102 E	380,415,470 240,395,435 270,310
3α:16α:17β-trihydroxy-5α-androstane	254-256	-5.9 M			
3α:16α:17β-trihydroxy-5β-androstane	274-278	+1.9 E	143-144.5	+43.5 E	

<u>Table 2.</u> C-21 steroids isolated from human urine.

Steroid	Melting point	$[\alpha]_D$		Melting point acetate	$[\alpha]_D$ acetate		H_2SO_4 Chromogen
5α-pregn-2-en-20-one	127-129	+132	E	-	-		
5β-pregnane-3α-ol	146						
5α-pregnane-3:20-dione	199-200	+117	E	-	-		
5β-pregnane-3:20-dione H	120-122	+112	C	-	-		237 (I)
3α-hydroxy-pregn-16-en-20-one H							
3α-hydroxy-5α-pregnan-20-one	174-175	+95.7	E	140-142	+112	E	
3α-hydroxy-5β-pregnan-20-one	148.5-149.5	+106	E	94-97			235, 331, 267
3α-hydroxy-5β-pregnan-17-iso-20-one	143-145	-38.9	E	154-157			
3β-hydroxy-5α-pregnan-20-one	194-195	+91.2	E	144-146	+77	C	234, 329, 228, 263
3β:20α-dihydroxy-pregn-5-ene T	184-185	-53.5	C	146-147	-56	C	
5α-pregnane-3α:20α-diol	248-248.5	+17	C	141.5-142.5	+18.8	C	343,430 256,391 322,502
5β-pregnane-3α:20α-diol	236-238	+27	E	176.5-178	+ 41	A	340, 431 258, 389 233, 323, 497
5α-pregnane-3β:20α-diol	211-212	+ 23	C	161	-2	E	320, 420 260, 380
5β-pregnane-3β:20α-diol H	182						

Table 2 contd.

Steroid		Melting point	$[\alpha]_D$		Melting point acetate	$[\alpha]_D$ acetate		H_2SO_4 Chromogen
3α-hydroxy-5β-pregnane-11:20-dione		174.5-175	+111	E	134-135	+135	C	319,401 262, 350 382, 414
3β:17α-dihydroxy-pregn-5-en-20-one	T	271-273	-37	D	234-235	-40	D	310, 410, 495 365, 460
3β:17aα-dihydroxy-17α-methyl-D-homo-androst-5-en-17-one	T	293-299	-105	C	273-278	-101	D	
3β:21-dihydroxy-pregn-5-en-20-one		139-159			164-165	+28	C	399, 378, 406, 476 255, 352, 390, 437 314, 533
3α:6α-dihydroxy-5α-pregnan-20-one		195-196	+105	E	155-156	+92.3	E	
3α:6α-dihydroxy-5β-pregnan-20-one		192-194	+66.3	C	128-129			
3β:6α-dihydroxy-5α-pregnan-20-one		206-210	+99	C	102-104	+74.5	C	
3α:20α-dihydroxy-5β-pregnan-11-one		214-217	+56.7	E	231-233	+56.7	E	
3α:17α-dihydroxy-5α-pregnan-20-one		214-216.5	+45.2	E	216.5-220	+1.7	C	
3α:17α-dihydroxy-5β-pregnan-20-one	H.T.	219-219.5	+64.5	E	201-202			238, 315, 410 225, 258, 379 327
3α:21-dihydroxy-5β-pregnan-20-one (Tetrahydrodeoxy corticosterone)	H							

Table 2 contd.

Steroid		Melting point	$[\alpha]_D$	Melting point acetate	$[\alpha]_D$ acetate	H_2SO_4 Chromogen
3β:16α:20α-trihydroxy-pregn-5-ene	T	245-248		178-180	-101 E	
3β:17α:20α-trihydroxy-pregn-5-ene	T	221-224		203-206	-79 E	
5α-pregnane-3β:16α:20α-triol		254-256		177-180	+54.1 E	
5β-pregnane-3α:17α:20α-triol		248-249		148-149 158-159		
21-hydroxy-pregn-4-ene-3:11:20-trione (11-Dehydrocorticosterone)		177-180	+299 E	179-181	+239 D	283, 354, 415 233, 323, 371 401
3α:17α-dihydroxy-5β-pregnane-11:20-dione		207-208	+68.5 A	208-209	+84 A	
11β:21-dihydroxy-pregn-4-ene-3:20-dione (Corticosterone)		177-180	+222 E	145, 153	+195 A	241, 287, 331, 374, 418,460 230, 247, 318, 367, 407, 432
3α:21-hydroxy-5β-pregnane-11:20-dione				82-90		
3α:17α:20α-trihydroxy-5β-pregnan-11-one	H	191-193 208-210	+19 A	225-226	+18.6 A	
3α:11β:21-trihydroxy-5α-pregnan-20-one (alloTetrahydrocorticosterone)		204-206		174	+84 C	

Table 2 contd.

Steroid	Melting point	$[\alpha]_D$	Melting point acetate	$[\alpha]_D$ acetate	H_2SO_4 Chromogen
3α:11β:21-trihydroxy-5β-pregnan-20-one (Tetrahydrocorticosterone)					
3α:17α:21-trihydroxy-5β-pregnan-20-one	214-216	+60 E	205-206	+88 E	
5β-pregnane-3α:17α:20:21-tetrol					
11β:21-dihydroxy-18-al-pregn-4-ene-3:20-dione (Aldosterone)	112, 166	+152.2 A	21Ac 198-199 18Ac 217-219	+121.7 C	288
17α:21-dihydroxy-3β-pregnane-3:11:20-trione (Dihydrocortisone)	233-235		228-230	+82 A	251, 270, 342, 421 248, 257, 292, 372 233, 242
17α:21-dihydroxy-pregn-4-ene-3:11:20-trione (Cortisone)	227-229	+204 E	240-242	+170 C	283, 342, 415 228, 319, 371 486
11β:17α:21-trihydroxy-pregn-4-ene-3:20-dione (Cortisol)	212-215	+168 E	220-224	+152 A	237, 282, 393, 475 229, 252, 352, 417
3α:17α:21-trihydroxy-5β-pregnane-11:20-dione (Tetrahydrocortisone)	194-198		235-238	+89 A	271, 340, 415 252, 290, 371 475

Table 2 contd.

Steroid	Melting point	$[\alpha]_D$	Melting point acetate	$[\alpha]_D$ acetate	H_2SO_4 Chromogen
3α:11β:17α:21-tetrahydroxy 5β-pregnan-20-one (Tetrahydrocortisol)			209-211	+90 C	332, 405, 506 275, 365, 488
3α:17α:21-tetrahydroxy-5β-pregnan-11-one (Cortolone)	208-209	+34.2 E	214-216	+28 C	
3α:17α:20β:21-tetrahydroxy-5β-pregnan-11-one (β-Cortolone)	260-261.5	+40 E	205-206	+87.3 C	
5β-pregnane-3α:11β:17α:20α:21-pentol (Cortol)	250.5-254	+23.7 E	168-170	0 C	
5β-pregnane-3α:11β:17α:20β:21-pentol) (β-Cortol)	262-264.5	+33.3 E			239, 414, 500 225, 323, 489 290, 360, 378, 446
6β:11β:17α:21-tetrahydroxy-pregn-4-ene-3:20-dione (6β-Hydroxycortisol)	239-241	+76 M	122-125	+76 M	

Abbreviations:- A = acetone, C = chloroform, D = dioxane, E = ethanol, M = methanol.

H - urine obtained from patients with adrenal hyperplasia.
T - do. do. do. carcinoma.

In column 6 (H_2SO_4 chromogen using the method of Zaffaroni, 1950):-
1st row shows position (mμ) of maxima.
2nd row do. minima.
3rd row do. inflections.

Table 14. Steroids containing a 5-en-3β-ol group, or artifacts derived from them, which have been isolated from urine.

Steroid	Source	m.p. °C.	$[\alpha]_D$	Acetate m.p. °C.	Acetate $[\alpha]_D$	Spectrum of sulphuric acid solution
Dehydroepiandrosterone (3β-hydroxy-androst-5-en-17-one)	Urine - normal (1,2,3,4,5,6)	137-138, 145-146 (4)	+10.9 E (1)	168-169 (8)	+3.9 E (1)	Acetate Max.:304,404,482 Min.:250,344,456 (27)
	Urine - adr. carcinoma (7,8,9,28)	139.5	+ 2 C (13)		-7 C (13)	
		147.5-149 (9)				
	Urine - adr. hyperplasia (10)					
	Urine - pregnant mare (11)					
	Urine - guinea pig (a) after admin. of androst-5-en-3β:17β-diol (6,12)					
	(b) after admin. of dehydroepiandrosterone (6)					

Table 14. contd.

Steroid	Source			Acetate		Spectrum of sulphuric acid solution
		m.p. °C.	$[\alpha]_D$	m.p. °C.	$[\alpha]_D$	
3β-chloro-androst-5-en-17-one	Urine - normal (1,4,5,15)	156-158 (9)	+19 E (16)	-	-	
	Urine - adr. carcinoma (8,9,16,28)		+15.5 C (15)			
	Urine - adr. hyperplasia (10)					
	Urine - after admin. of androst-5-en-3β:17β-diol (6)					
Androst-3:5-dien-17-one	Urine - normal (4)	78-84 (4)		-	-	
	Urine - after admin. of testosterone (17)					
3:5-cycloandrostan-6β-ol-17-one (i-Androstanolone)	Urine - normal (18)	140-141 (18)	+121 E (18) +111 C (19)	109-111 (18)	+117 E (20)	

Table 14 contd.

Steroid	Source	m.p. °C.	$[\alpha]_D$	Acetate m.p. °C.	Acetate $[\alpha]_D$	Spectrum of sulphuric acid solution
Androst-5-ene-3β:17β-diol	Urine - adr. carcinoma (16,21,22,28)	155 178-182 (21)	-53.2 E (16)	3-acetate 147-148 (25)	-55.8 E (26)	Max.:240,307,404,482 Min.:233,255,348,472 Infl.: 440,540 (27)
	Urine - after admin. of dehydroepiandrosterone (23)		-61 C (24)	17-acetate 146-147 (27)	-69 C (27)	
				Di-acetate 158-160 (21) 165-166 (25)	-54 E (27)	
3β-chloro-androst-5-en-17β-ol	Urine - after admin. of $CH_3C^{14}OONa$ (28)	165-168 (28)		185-187 (28)		
3β-hydroxyandrost-5-ene-7:17-dione	Urine - adr. tumour (29)	245-248 (29)	-66.4 M (29)	184.5-187.5 (29)	-72 E (29)	

Table 14 contd.

Steroid	Source	m.p. °C.	$[\alpha]_D$	Acetate m.p. °C.	Acetate $[\alpha]_D$	Spectrum of sulphuric acid solution
3β:16α:17β-trihydroxy-androst-5-ene	Urine - normal (30) Urine - adr. tumour (16,31,32)	265-270 (31) 261-264 (32)	-88.5 D (30) -75.7 A (30)	3-acetate 243-245 (31) diacetate 183-187 (31) triacetate 189-191 (30)	 -102 E (31)	Max.:380,415,470 Min.:240,395,435 Infl.:270,310 (14)
3β:20α-dihydroxy-pregn-5-ene	Urine - adr. tumour (21,22,28) Urine - pregnant mare (34)	184-185 (21)	-53.5 C (35)	3-acetate 143.5-145 (36) 20-acetate 167-169 (36) 3:20-diacetate 137 146-147 (21)	 -48 E (36) -62 C (37) -54 C (37) -45 E (38) -56 C (37)	

Table 14 contd.

Steroid	Source	m.p. °C.	$[\alpha]_D$	Acetate m.p. °C.	Acetate $[\alpha]_D$	Spectrum of sulphuric acid solution
3β:17α-dihydroxy-pregn-5-en-20-one	Urine - adr. tumour (9)	271-273 (39)	-37 D (39)	3-acetate 234-235 (39) 17-acetate 230-231.5 (40) Diacetate 180-182 (40)	 -40.9 D (39) -68 D (40) -59 C (41) -64.9 D (40)	Max.:310,410,495 Min.:365,460 (33)
3β:17aα-dihydroxy-17aβ-methyl-D-homoandrost-5-en-17-one	Urine - adr. tumour (9)	302-305 (42)	-110 D (42)	277-279 (40)	-101 D (42)	
3β:16α:20α-trihydroxy-pregn-5-ene	Urine - adr. tumour (38)	245-248 (38)		16:20 diacetate 185.5-187.5 (38) Triacetate 178.5-180 (38)	 -101 E (38)	

Table 14 contd.

Steroid	Source	m.p. °C.	[α]D	Acetate m.p. °C.	Acetate [α]D	Spectrum of sulphuric acid solution
3β:17α:20α-trihydroxy-pregn-5-ene	Urine - adr. tumour (21, 43)	221-224 (43)		3:20 diacetate 203-206 (43)	-79 E (43)	
3β:21-dihydroxy-pregn-5-en-20-one	Urine - adr. tumour after admin. of ACTH (44)	139-159 (45)		21-acetate 184-185 (45)		Diacetate Max.:339,378,406,476 Min.:255,352,390,437 Infl. 314,533 (27)
				3-acetate 161-162 (46)	+192 E +182 C (46)	
				Diacetate 165-166 (47)	+28 C (47)	

Abbreviations: A = acetone, C = chloroform, D = dioxane, E = ethanol M = methanol

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