AN INVESTIGATION OF THE URINARY CORTICOSTEROID PATTERN IN ADRENAL CORTICAL DISEASE BY THE TECHNIQUE OF PAPER CHROMATOGRAPHY

by

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Thesis

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INTRODUCTION

The purpose of this investigation was to study the urinary corticosteroid pattern in adrenal cortical and anterior pituitary disease. Patients with both adrenal cortical hyperfunction and hypofunction were investigated in the untreated disease state, after hormonal therapy and also in two cases after surgical treatment. Normal subjects were studied for purposes of comparison. The qualitative and quantitative differences in the patterns were thus evaluated under various physiological conditions.

While this investigation was in progress the results of the recent work of other investigators have been published demonstrating the usefulness of the paper chromatographic technique for the separation of the urinary corticosteroids.

A study of the paper chromatographic patterns together with the use of chemical and physical technique for identification has shown the nature of the component steroids. Qualitative differences in the patterns have been demonstrated in some pathological states which may in some cases help to reveal the nature of the disease. For instance, such studies on congenital adrenal hyperplasia have given valuable

information as to the nature of the abnormality of adrenal cortical hormone synthesis pointing to a defect involving a deficiency of one or more hydroxylating enzymes.

In addition a picture of the relative amounts of the various corticosteroids making up the pattern is obtained. Accurate assays for specific functional groupings of the steroid molecule have been developed but these do not give any information about the relative proportion of any one metabolite.

L I T E R A T U R E R E V I E W

PART ONE

THE NATURE AND FUNCTION OF THE ADRENAL CORTICAL HORMONES

I. Studies on "Cortin"

Extracts of the adrenal cortex were first prepared in 1930 by Hartman and Brownell and Swingle and Phiffner. These extracts were shown to have potent biological activity when administered to adrenalectomized laboratory animals and were called "cortin". The name became applied as well to those unfractionated urinary substances that possessed biological properties demonstrable with adrenal cortical extracts and pure adrenal cortical compounds.

Further intensive investigation led to the fractionation of this "cortin" material and to the isolation from adrenal cortical and urinary extracts of a considerable number of biologically active and largely inactive compounds.

After exhaustive crystallization the amorphous residue still had activity in life maintenance of 14 to 30 per cent of the original crude extract (118, p. 1272). In 1953, aldosterone, the highly potent mineralocorticoid, was first isolated from the amorphous fraction (75).

II. Steroids Isolated from Adrenal Cortical Tissue

Since for obvious reasons no such intensive investigations can be carried out on non-pathological human adrenals, bovine and hog tissues have been studied. The C21 steroids isolated from bovine adrenal cortical tissue are listed in Table I.

The corticosteroids have the cyclopentanoperhydrophenanthrene nucleus which is characteristic of the steroid molecule and an additional two carbon side chain at position 17.

A study of the table shows that

(a) a relatively high proportion of the compounds have the Δ^4-3-ketone grouping. These steroids include:

(i) the biologically active compounds, F, E, B, A, DOC and progesterone;

(ii) precursors of the above hormones.

(b) the compounds with reduction at ring A have with one exception the 3β-hydroxyl, allopregnane, (5α) configuration. These are mainly biologically inactive metabolites.

TABLE I

C_{21} STEROIDS ISOLATED FROM ADRENAL TISSUE (BOVINE)

No.	Structure	Series	
1.	Allopregnane-3β,11β,17α,20,21-pentol	$C_{21}O_5$	
2.	Allopregnane-3β,11β,17α,21-tetrol-20-one		
3.	Allopregnane-3α,11β,17α,21-tetrol-20-one		
4.	Allopregnane-3β,17α,21-triol-11,20-dione		
5.	Δ^4-Pregnene-11β,17α,20β,21-tetrol-3-one		
6.	Δ^4-Pregnene-17α,20β,21-triol-3,11-dione		
7.	Δ^4-Pregnene-11β,17α,21-triol-3,20-dione (F)		
8.	Δ^4-Pregnene-17α,21-diol-3,11,20-trione (E)		
9.	Allopregnane-3β,17α,20β,21-tetrol	$C_{21}O_4$	(a) 11-desoxy group
10.	Allopregnane-3β,17α,21-triol-20-one		
11.	Δ^4-Pregnene-17α,21-diol-3,20-dione		
12.	Allopregnane-3β,11β,21-triol-20-one		(b) 17-desoxy group
13.	Allopregnane-3β,21-diol-11,20-dione		
14.	Δ^4-Pregnene-20β,21-diol-3,11-dione		
15.	Δ^4-Pregnene-11β,21-diol-3,20-dione (B)		
16.	Δ^4-Pregnene-21-ol-3,11,20-trione (A)		
17.	Allopregnane-3β,17α,20β-triol	$C_{21}O_3$	
18.	Allopregnane-3β,17α,20α-triol		
19.	Allopregnane-3β,17α-diol-20-one		
20.	Δ^4-Pregnene-17α-ol-3,20-dione (17-OH progesterone)		

TABLE I (continued)

No.	Structure	Series
21.	Δ^4-Pregnene-21-ol-3,20-dione (DOC)	
22.	Allopregnane-3β-ol-20-one	$C_{21}O_2$
23.	Δ^4-Pregnene-3,20-dione (progesterone)	

III. Extra-Adrenal Sources of the Adrenal Cortical Hormones

1. Isolation from Other Tissues

Glucocorticoids have been isolated in relatively small amounts from human placental tissue. This might explain the ability of Addisonian women to survive pregnancy and the increased corticosteroid levels observed in normal pregnancy by Venning (120, p. 8).

No corticosteroids have been obtained from the ovary, testes or pituitary (120, p. 8).

2. Corticosteroids Found in Blood

Pincus and Romanoff (116) have analysed human adrenal venous blood of cancer patients treated with ACTH and have isolated hydrocortisone, corticosterone, tetrahydrocortisone and two 17-ketosteroids, 11β-hydroxy-Δ^4-androstene-3,17-dione and Δ^4-androstene-3,17-dione. They also detected these five metabolites in human peripheral blood.

Morris et al.(73) have demonstrated the presence of corticosterone and 11-dehydrocorticosterone as well as hydrocortisone and cortisone in human peripheral blood.

3. Corticosteroids Found in Urine

These are reviewed in detail in Part Three. However, briefly, they include the following types of compounds:

(a) The Free Fraction

A small proportion of the corticosteroids is excreted in the unaltered or free state. The Δ^4-3-ketone, α,β-unsaturated grouping is intact and the biological activity is retained. Compounds F, E, B and aldosterone have been isolated.

(b) The Conjugated Fraction

The majority of the urinary corticosteroids are excreted as conjugates. Conjugation of the molecule with another compound results in considerable reduction of its original biological activity.

The active adrenal cortical hormones are reduced in the following possible ways:

(i) Reduction only of the 3 carbon ketone to either the 3 alpha or 3 beta hydroxyl group to form the so-called "dihydro"-pregnene derivative;

(ii) Reduction only of the double bond between carbon atoms 4 and 5 to form the so-called "dihydro"-pregnane (5β) or allopregnane (5α) derivatives;

(iii) Reduction of the 3-ketone to either the 3α or 3β hydroxyl group, together with saturation of the Δ^4 group to either the pregnane or allopregnane configuration, to form the so-called "tetrahydro" derivative.

Reduction is followed by conjugation at the 3 carbon hydroxyl (79). Conjugation occurs largely in the liver (79).

IV. Functions of the Adrenal Cortex

They are the following:

1. Life maintenance (109) (1) (120, p. 9).
2. Control of electrolyte metabolism (75) (120, p. 9).
3. Influence on carbohydrate metabolism (107) (106) (120, p. 9).
4. Influence on protein metabolism (106) (120, p. 9).
5. Important influence on all forms of stress (109) (123).
6. Properties of sex hormones (110).

V. The Relation of Chemical Structure to Biological Activity

Certain configurations are essential for potent biological activity of any type (118, p. 1275).

1. The α,β-unsaturated, Δ^4-3-ketone grouping.

2. The presence of a primary hydroxyl group at carbon 21 and a ketonic oxygen at carbon 20 -- the alpha-ketolic side chain is necessary for all activity except that of life maintenance.

3. The isomer at carbon 17 of the side chain must have the α configuration.

Certain additional functional groups are directly related to specific physiological activity, although any one of the biologically active compounds if administered in sufficiently large doses will have some influence on all aspects of adrenal cortical hormone function.

1. The presence of an oxygen, ketonic or alcoholic, grouping at carbon 11 enhances activity in the regulation of carbohydrate metabolism.

e.g. compounds F, E, B and A (glucocorticoids).

2. A hydroxyl group at carbon 17 in addition to oxygenation at carbon 11 still further enhances activity in carbohydrate metabolism regulation.

e.g. compounds F and E are more powerful glucocorticoids than are compounds B and A.

3. The absence of an oxygen atom at carbon 11 may endow the molecule with greater activity in the regulation of electrolyte and water metabolism.

e.g. compounds DOC and S (mineralocorticoids). However, the most potent mineralocorticoid known, aldosterone, does have an oxygen atom at carbon 11.

PART TWO

A REVIEW OF THE METHODS

I. The Hydrolysis and Extraction of Urinary Corticosteroids

1. Extraction Without Prior Hydrolysis

(a) The Free Fraction

The free fraction is chloroform extractable at neutral pH without prior hydrolysis. About 60 gamma or more of Porter-Silber chromogenic corticosteroids are excreted daily. These include mainly hydrocortisone and cortisone which in normal urine are extracted in greatest part from the free fraction.

(b) The Conjugated Fraction

The intact conjugates may be extracted very efficiently with butanol.

A number of investigators prefer to work on the conjugated corticosteroids directly, without any hydrolysis, e.g. butanol extracts have been subjected to chemical assay (83) and paper chromatographic resolution (88).

2. Methods of Hydrolysis

It has been shown (68) that heating acidified urine destroys reducing and glycogenic activity and also that glucuronides are resistant to alkali and mild acid treatment.

Consequently, most investigators use either cold strong acid hydrolysis or enzyme hydrolysis or both.

(a) Acid Hydrolysis

The acid hydrolysis is commonly carried out at room temperature with the pH of the urine adjusted to 1.0.

The length of time for which the urine specimen is allowed to stand at pH 1.0 is very important (68) (121).

(i) Extraction immediately after acidification.

The amount of extractable corticosteroids is about double the amount of the free titre when measured by the liver glycogen deposition test (121).

(ii) Extraction after two hours.

A decrease of 75 per cent was observed by Patterson and Marrian (68).

(iii) Extraction after 24 hours.

After the first few hours there is a slow rise of formaldehydogenic steroids which at the end of 24 hours is at a higher level than the initial one (121) (68).

(iv) Extraction after a few days.

Acidified urine was allowed to stand at room temperature for 1 to 11 days. After the first day, no further decrease was observed, while the level of increase, if any, varied considerably in different specimens (68).

(b) Enzyme Hydrolysis

The most efficient method for the hydrolysis of glucuronide conjugates is enzyme hydrolysis. Beta-glucuronidase of both animal and bacterial origin is now prepared commercially and is widely used. Some investigators have prepared their own β-glucuronidase extracts (9). Beef spleen or calf liver extracts function best at a pH range of 4.8 to 5.0 and the optimum temperature is 37° C. Bacterial source β-glucuronidase functions best at pH 6.0 and is also used at 37° C. (68).

Incubation time varies from one to three or four days. A considerable excess (two or three hundred units or more of enzyme activity per ml. of urine) of β-glucuronidase is commonly used. As Mason (68) has pointed out, this excess may be necessary if,

(i) the glucuronic acid that is released is inhibitory,

(ii) there are other inhibitory factors in the urine,

(iii) there are other substrates in the urine which are split by the enzyme resulting in a state of competitive inhibition.

3. The Types of Conjugates Liberated by Acid Hydrolysis

The above hydrolysis experiments and others indicate that there is more than one type of conjugate present in the urine.

There are those conjugates present in small amounts which are readily hydrolysed by acid and yield corticosteroids which are acid labile and therefore destroyed by the acid as soon as they are liberated. These might include any sulphates as shown by Lieberman and Dobriner (121).

There are those conjugates present in large amounts which are less readily hydrolysed by acid but which yield acid stable corticosteroids. These are mainly the glucuronides.

There is also evidence for the presence in urine of a conjugate extractable with chloroform at pH 1.0 but not at neutral pH. It is not split by β-glucuronidase hydrolysis but it is hydrolysed at pH 3 to 4 in 1 to 3 hours (121). This unknown conjugate apparently yields both acid stable and acid labile formaldehydogenic steroids (68).

Compounds with the Δ^4-3-ketone grouping intact, such as cortisone, have been recovered in the conjugated fraction in varying amounts. In the extraction of a pregnancy urine De Courcy and Gray (122) obtained most of the cortisone only after acid hydrolysis. However, some investigators feel that such compounds cannot be conjugated at the 3 carbon position. Perhaps conjugation for excretion can occur at another part of the molecule.

4. The Importance of β-glucuronidase Hydrolysis

(a) The development of enzyme hydrolysis has made it possible to determine routinely urinary corticosteroid patterns in one to three day volumes of urine, whereas previously many litres of urine were required for the isolation and identification of certain steroid components.

(b) Enzyme hydrolysis is not only more efficient but it is probably more safe than acid hydrolysis.

(c) Its use in the clinical laboratory is of importance for it is now possible to do routine determinations on the total corticosteroid levels in about 25 c.c. of urine.

5. The Efficiency of Hydrolysis

The results of Dr. Wilson (68 - Discussion) have led her to conclude that the yield of corticosteroids is very much dependent upon the number and the thoroughness of the extractions as well as upon the length of incubation time. (This suggests that perhaps during hydrolysis a point is reached where there is an equilibrium between the liberated steroids and the conjugates and that the removal of some free steroids is necessary for any further splitting.) This is in apparent contrast to the hydrolysis of 17-ketosteroids which is more a function of time.

6. The Possibility of Artifact Formation

Artifact formation may result as a consequence of hydrolysis and extraction procedures and distort the true picture of the urinary steroid metabolites. Artifact formation as a result of substitution, dehydration or other reactions is not likely to occur during the milder hydrolysis procedures used for corticosteroid studies as it is during the more vigorous hydrolysis techniques employed for the isolation of 17-ketosteroids (120, p. 22).

However, some artifacts may appear as a result of reactions of rearrangement of the functional groupings on the side chain at position 17.

For instance, Hirschmann and Hirschmann (120, p. 25) have detected two compounds, Δ^5-pregnene-3β,17α-diol-20-one and 17α-methyl-Δ^5-D-homoandrostene-3β,17α-diol-17-one, in the same urine. The conversion into the latter compound has been obtained by acid and alkali treatment.

Although it is not regarded as an artifact, steroids extracted in the free fraction may also include compounds which were originally excreted as conjugates. However, due to the presence of endogenous β-glucuronidase (especially in late toxemia of pregnancy), there is splitting of the conjugates. The addition of saccharolactone to the urine after collection inhibits this activity.

II. The Purification of the Crude Urinary Extract

1. Preliminary Purification

Washing with cold alkali is commonly used to remove most of the extraneous pigment as well as the acid and phenolic steroid fractions from the crude extracts. The remaining neutral fraction is then washed with distilled water.

For most normal urine extracts this treatment together with some purification achieved during paper chromatography is sufficient to obtain clean, well developed chromatograms. However, some extracts of pathological urines, as in liver disease and toxemia of pregnancy, are not sufficiently cleaned.

2. Techniques for Further Purification

The method and degree of further purification should depend upon the nature of the investigation and whether qualitative or quantitative results are desired. The following methods have been developed which in some cases effect fractionation as well.

(a) Girards Reaction

In the mid thirties Girard and Sandulesco (120, p. 149) introduced the use of the reagents T (trimethylaminoaceto-hydrazide hydrochloride) and P (pyridylacetohydrazide hydro-chloride)for the separation of ketonic from non-ketonic compounds. The ketones are converted into hydrazone derivatives

by a simple procedure which has been used by many investigators as the initial step in the fractionation of steroid mixtures (19) (39) (91).

(b) Chromatography

(i) Paper chromatography

Zaffaroni (17) (18) (114) has published techniques of paper chromatography in which most of the extraneous pigment and fatty material present in the crude alkali washed extract is removed and a rough fractionation into $C_{21}O_3$, $C_{21}O_4$ and $C_{21}O_5$ metabolites is achieved simultaneously.

(ii) Column (adsorption) chromatography

Silica gel: Dr. Romanoff (85) (86) is one of a number of workers who has used silica gel chromatography to purify glucuronidase hydrolysed extracts and has found that 70 per cent by weight of non-steroidal pigment material was efficiently removed. Briefly, the extract was dissolved in some benzene and transferred to a benzene wetted micro column. The column was then developed by running through a series of solvents starting with benzene, then benzene-ether (in varying proportions), ether, ether-ethylacetate (in varying proportions), acetone and finally absolute methanol was used. Thus, many separate fractions of corticosteroids were obtained which were arbitrarily combined into three main fractions on the basis of polarity.

Engel (38) using mixtures of cyclohexane-ethylacetate,

and Neher (75) using mixtures of chloroform-acetone have also used silica gel columns.

Florasil: The 17-ketosteroids are eluted with 2 to 4 per cent methanol in chloroform while the corticosteroids are eluted with 25 per cent methanol in chloroform (37) (44) (87).

Magnesium silicate : celite (1 : 1): The 17-ketosteroids are eluted with 2 per cent ethanol in chloroform while the corticosteroids are selectively eluted with 15 per cent ethanol in chloroform (126).

In general, this technique is efficient but tedious and simultaneous studies on the recovery of standards must be carried out to check the losses involved.

(c) Partitioning Between Two Solvent Phases

(i) Benzene-water partitioning

The following modification is that of De Courcy et al. (31).

1. The crude residue is dissolved in 0.1 ml. of methanol and 10 ml. of benzene.
2. This is extracted ten times with 10 ml. of water.
3. The water phase is extracted four times with 25 ml. of chloroform which is then evaporated down. This is the so-called "water fraction" and contains the more polar metabolites.
4. The benzene phase is called the "benzene fraction" and according to De Courcy contains no alpha-ketolic

material. Gray has found benzene-water partitioning eliminates the measurement of the least polar corticosteroids (49).

The technique is widely used (29) (43).

(ii) Alcohol-petroleum ether partitioning

A number of workers use partitioning between alcohol and petroleum ether to remove much fatty material and pigments from crude extracts, e.g. Neher (75), Pechet (78), Burstein (19), Schneider (91).

(d) Ion Exchange Resins

Ion exchange resins have been used to purify alkali washed extracts prior to application on paper, by Gulyassy (51).

(e) Charcoal Adsorption

This relatively simple technique has been used by some investigators to remove much urinary pigment. It is not widely used due to the fact that artifact formation was reported when Soxhlet extraction was used to remove corticosteroids from an adsorbing charcoal mixture.

However, Lombardo (62) has recently described a technique which employs activated charcoal to decolorize a 24 to 48 hour urine extract at room temperature, with safety. The author used over 150 c.c. of 40% benzene in absolute ethanol for elution and obtained a quantitative recovery of corticosteroids with no apparent artifact formation.

III. The Fractionation of Urinary Corticosteroids

1. The Technique of Paper Chromatography

(a) History

Schonbein at about 1860 noted that when an aqueous solution is applied to filter paper strips the water ascends the paper faster than the solute in solution, and if the solute is a mixture, one of the components ascends much faster than any of the others. This technique of "Kapillaranalyse" was later applied to the separation of fats, alkaloids, plant pigments and other compounds. Unlike the technique of paper partition chromatography which was developed about 80 years later, no developing solvent was used (125, p.1).

In 1944, Consden, Gordon and Martin first effected a successful separation of a mixture of amino acids by the technique of descending paper partition chromatography replacing silica gel, which they had previously used, as an inert support (125, p.1).

(b) Theory and practise

The phenomenon of paper chromatography depends upon the partitioning of solute applied to filter paper between two phases, so that the partition coefficient of a component steroid between two solvent systems - one of them moving with respect to the other - determines its chromatographic behaviour in the system.

In all systems of paper chromatography certain rules are followed:

(i) The composition of the mobile phase must be kept constant throughout development. Therefore chromatography is carried out in an enclosed chamber which is saturated with both mobile and stationary phases at constant temperature.

(ii) The developing solvent should move at a relatively slow rate (2 to 3 centimeters per hour).

(iii) The components to be separated should have small but distinctly different partition coefficients in the solvents used.

Solute in solution in methanol or other solvent is applied either as a band or as a spot at some distance from one end of the filter paper strip. The paper is then placed in a jar for development. Mobile phase flows past the solute mixture and down the length of the paper by capillary action.

The rate of movement of an individual steroid in any solvent system may be expressed as its absolute R_F value in that system if development of the papergram is stopped before the solvent front flows off the paper (125, p. 9) (22).

The R_F value for a separated compound is:

$$R_F \quad \frac{\text{movement of band (distance from the starting line to mid band)}}{\text{movement of advancing front of liquid (from the starting line)}}$$

The rates of movements of the corticosteroids in the Zaffaroni systems (18) are dependent upon:

(i) The number and the nature of oxygen functions on the molecule.

(ii) The hydroxyl group exerts a greater polar effect than does a ketonic oxygen in the same position.

(iii) In general, the more polar the compound, the slower its rate of movement.

(c) Application to the separation of the free and hydrolysed corticosteroids.

(i) Early developments

The group of Burton, Zaffaroni and Keutman were the first to apply paper chromatography to the separation of corticosteroids extracted from adrenal cortical tissue and urine, after hydrolysis (15) (17) (18).

They initially tried to resolve the ketonic fraction after it was separated by the Girard reaction. The hydrazone derivatives were then applied to paper and chromatographed. The technique was not successful because 1), the hydrazone derivatives had running rates too similar to allow separation and 2), each component steroid reacted to form both the mono and the dihydrazone derivatives and thus yielded two spots on the paper.

The authors found that in the benzene-water systems separation was obtained but with "tailing" or streaking. Bush (22) also found that water was not a satisfactory stationary phase because the steroids tended to move with the solvent front.

There developed as a result two systems of paper chromatography which differ mainly in the nature of the stationary phase employed. Both are methods of descending chromatography.

(ii) The Zaffaroni solvent systems

The Zaffaroni systems employ no water. Instead, the paper is impregnated prior to use with an organic solvent such as formamide or propylene glycol (diluted or undiluted with methanol). This provides a non-volatile stationary phase.

Equilibration between the two liquid phases is achieved by saturating the mobile phases, toluene and benzene, with propylene glycol and formamide, respectively, and development is carried out at room temperature.

Since their introduction these two solvent systems have been used very widely. The resolving power of the toluene-propylene glycol system is more satisfactory for the $C_{21}O_5$, most polar steroids, whereas the benzene-formamide system is best for the preliminary fractionation of the crude extracts, for the resolution of the $C_{21}O_3$ steroids and for the chromatography of acetyl and other ester derivatives.

The length of development, anywhere from 6 hours to about 7 days, depends upon the types of compounds to be separated, the solvent system used and upon the amount of mobile phase used.

(iii) The Bush solvent systems.

Bush (22) found that the addition of isopropanol to the water/hydrocarbon mixture eliminates tailing. He devised systems employing non-polar solvents as the mobile plase and aqueous methanol as the stationary plase.

Equilibration between the developing solvent and the stationary phase occurs at elevated temperatures (30° to $38^{\circ}C.$) via the vapor phase and chromatography is carried out at the same temperature.

The most polar corticosteroids, down to the $C_{21}O_3$ compounds, can be separated to some extent on one paper in a few hours time and absolute R_F values can be calculated.

(iv) A comparison of these systems

The following is a summary of the Zaffaroni and Bush methods (21):

	Advantages	Disadvantages
Zaffaroni - paper impregnated with non-volatile solvents	1. Large volumes of material easily handled. 2. Very non-polar steroids easily handled. 3. Very crude extracts usually easily handled.	1. Displacement effects. 2. Lengthy drying. 3. No R_F values. 4. R_T values usually have large errors. 5. Long running time.
Bush - orthodox method with volatile solvents	1. Displacement effects are small or absent. 2. Chromatograms are easily dried. 3. R_F values obtained. 4. Short running times. 5. Resolution per unit running time is in most cases better. 6. Very low background in spectrophotometer	1. Careful temperature control. 2. Large quantities not as easily handled. 3. Steroids less polar than C_{21}-diones are not well characterized.

(v) Other solvent systems in use

Since these original systems were described other investigators have devised new systems or modified the old ones.

Pechet (78) has developed a method which involves no pre-impregnation of the paper. Development time is very short (two hours) and at room temperature. Water is the stationary phase while the developing or mobile phase includes 20 volumes toluene; 10 volumes petroleum ether; 1.5 volumes butanol; 1.5 volumes ethanol, and 7 volumes of water.

Schwartz (93) has described a method which first utilizes the technique of ascending chromatography followed by further resolution for longer periods by the Zaffaroni method.

Eberlein and Bongiovanni (25) recently have described new modifications of the Bush system which permit chromatography at room temperature. Also several new systems which employ tertiary butanol alone, or diluted with methanol, as the stationary phase have been developed. Certain corticosteroids, including aldosterone, can be rapidly separated using these new systems alone but especially in combination with the Zaffaroni and Bush systems.

It is possible with the many solvent systems available to choose the appropriate one or more than one for the resolution of a certain steroid component, e.g. aldosterone (75).

(d) Paper Chromatographic Separation of Intact Steroid Conjugates.

Paper chromatographic methods are now being developed by Schneider et al. which effect the separation of steroids conjugated with free glucuronic and sulphuric acids or their Na salts (88).

Descending chromatography is carried out at 23° to 29° C. for 6 to 8 hours.

The solvent systems described are:

System I : n-butylacetate-n-butanol-10% acetic acid (80:20:100)

" II : n-butylacetate-n-butanol-10% formic acid (80:20:100)

" III: n-butylacetate-methanol-0.1 M Na barbital buffer in 50% methanol, pH 8.2 (150:50:50).

2. Non-Paper Chromatographic Technique

The following are techniques other than paper chromatographic for the fractionation and separation of adrenal cortical hormones and their metabolites.

(a) Column Chromatography

Reichstein in 1936 introduced the technique of adsorption column chromatography. Callow and Callow early (1939) used this method for the fractionation of urinary steroids (120, p. 149). Adsorption column chromatography of different kinds is still widely used.

(i) Silica gel (41) (86) (85).

(ii) Magnesium silicate : celite (91) (92).

(iii) Gradient elution chromatography:

The authors Morris and Williams (73) used the principle of gradient elution chromatography for the separation of blood corticosteroids, in which the nature of the gradient obtained is determined by the shape of the apparatus and is reproducible. The mobile phase solvents are continuously changed automatically with the weaker eluant always being replaced by a stronger one. The first mobile solvent was 25 per cent

light petroleum ether in toluene and this was continuously changed until 100 per cent ethylene dichloride was the final elutant.

(b) Counter-current Distribution

This very efficient but involved procedure depends upon the partition coefficient between two solvent phases and has been used by Engel in the corticosteroid field (38) (39). The ketonic fraction was subjected to a 150 transfer counter current distribution in 50 cyclohexane : 50 ethylacetate/ 30 ethanol : 70 water system and analysed.

IV. The Characterization and Determination of the Urinary Corticosteroids

1. Characterization and Identification

The following are techniques which are used in the interpretation of the urinary corticosteroid pattern.

(a) Ultraviolet Light Absorption

(i) Qualitative test

The study of the entire paper under ultraviolet light at about 240 mμ roughly establishes the positions of those steroids with the Δ^4-3-ketone, α,β-unsaturated grouping (85).

(ii) Quantitative test

A single corticosteroid spot is eluted and its absorbing maxima (in methanolic solution) are read from about 220 to 330 mμ in the Beckman spectrophotometer (85).

(b) Scanning Patterns

The entire length of a one centimeter wide strip of the papergram is removed and scanned at 240 mμ in a photelectric densitometer. Curves depicting areas of ultraviolet light absorbing compounds may be plotted.

The strip may then be treated with blue tetrazolium reagent (65) and scanned at 520 mμ in an identical manner. On superimposition of the two sets of curves a valuable step toward the characterization of those corticosteroids with the Δ^4-3-ketone and alpha-ketolic structure is obtained.

Richardson et al. (84) use this technique for the semi-quantitative estimation of the corticosteroids separated on the strip.

(c) Spot tests

The tests that have been developed for the detection of various chemical configurations on the steroid molecule are listed in Table 2.

(d) Mixed Chromatograms

This is a very simple and useful technique which was introduced by Zaffaroni et al. (17)(18). If an unknown compound is chromatographed along with a standard and has similar running rates in more than one system and even after acetylation it is presumed to be identical with the standard. Bush (21) has criticized the analogy of the mixed chromatogram to the mixed melting point of orthodox chemistry for he stresses that entirely different physicochemical properties are involved.

(e) Chromatography of Ester Derivatives

Acetates of unknown compounds and standards are prepared simply by treating the dried steroid with acetic anhydride and pyridine. Propionates are also easily prepared. These derivatives are non-polar and developed with very short running times in the benzene-formamide system (17)(18). Hence the presence of acetylable hydroxyl groups can be detected by the great increase in R_F values after acetylation when compared to that of the free compound.

TABLE 2.

SPOT TESTS FOR THE DETECTION OF VARIOUS URINARY C_{21} COMPOUNDS ON PAPERGRAMS

Reference	Reagent	Group Giving Reaction	Color or Conversion Product
Burton et al. (17)	alkaline $AgNO_3$	α-ketols	brown
"	" "	DOC	"
"	TPTZ	primary α-ketol	red
Nowaczynski et al. (128), micro	"	" "	"
"	BT	" "	blue
Burton et al. (17)	iodine	Δ^4-3-C= O	yellow
"	"	compound E	blue
"	potassium permanganate	1. Δ^4-3 -C= O 2. α-ketol 3. C_{17}:20:21-triols	brown
Block et al. (125, p.109)	phosphoric acid, heat	α-ketol	fluorescence
Bush (21)	methanolic NaOH, heat	Δ^4-3-C= O	yellow fluoresence under ultra violet light

(f) Absorption Spectra in Concentrated Acid

(i) Sulphuric and chromogens

Spectroscopy in concentrated H_2SO_4 is a reliable technique if all materials used are very pure and clean. The Zaffaroni technique (115) is the following:

Three ml. of concentrated H_2SO_4 are added to 70 to 90 gamma of the dried, pure steroid in a test tube. The tube is stoppered and allowed to stand at room temperature for two hours. The optical density of the solution from 220 μ to 600 mμ is then read in a Beckman spectrophotometer. Each compound forms specific chromogens with the concentrated acid and the absorption spectra can be obtained and compared with those already recorded in the literature by Bernstein (10), Wilson et al. (127), Romanoff (85) (86), and others.

(ii) Phosphoric acid chromogens

Nowaczynski (77) has developed a technique for the identification of steroids by measurement of their chromogenic spectra in 100% phosphoric acid after heating at 105° C. The optical densities of the solution are read from 220 to 600 mμ in a Beckman spectrophotometer. All the spectra obtained were different from those given by sulphuric acid treatment and in nearly all cases characteristic of each compound.

In both cases, the concentrated acid apparently splits off water and effects double bond formation in the corticosteroid molecule, thus rendering it chromogenic.

(g) Infra-red spectrophotometry

Furchgott (1946) and Dobriner (1948) were the first to apply this technique to the corticosteroid field (120, P. 149).

It is the most conclusive and objective method for the absolute identification of any compound. Each steroid has in a certain region of the infra-red a unique and charcteristic spectrum.

2. Chemical Assay of Corticosteroids

A number of very useful quantitative chemical tests have been devised which measure specific functional groupings on the steroid molecule. These chemical assays may be applied to washed unfractionated urinary extracts or to compounds that have been separated on paper chromatograms and then eluted with methanol. They are listed in Table 3 with information about the type of reaction, the reagents used, and the chemical configuration giving the reactions.

The Porter-Silber Reaction: Previously, the formaldehydogenic steroid determination was used routinely in the clinical laboratory. However, it was found to measure much non-corticosteroid material in the enzyme hydrolysed fraction (88).

The phenylhydrazine reaction of Porter and Silber is now extensively used. It is simple, relatively more specific than the periodate oxidation method, and is described below.

TABLE 3.

CHEMICAL ASSAYS

Author	Material Measured	Reagent	Type of reaction
Talbot et al. (98)	primary α-ketol	copper reagent in alkaline solution	reduction
Heard and Sobel (54)	Δ 4-3C=O α-ketol or both	phosphomolybdic acid	reduction
Mader and Buck (65) Chen and Tewell (24) Nowaczynski et al. (128)	primary α-ketol	blue tetrazolium triphenyl tetrazolium Cl. (128)	reduction
Daughaday et al. (29)	primary α-ketol dihydroxy acetone glycerols	periodic acid	oxidation and liberation of formaldehyde
Cox (28)	C_{21}-17:20-diols -21-desoxy	periodic acid	oxidation and liberation of acetaldehyde
Gornall et al. (47)	ketone at various positions e.g. C_3,20	2,4-dinitrophenylhydrazine	forms a phenylhydrazone
Porter and Silber (81)(129)	dihydroxy acetone i.e. 17:21-diol-20-ketone	phenylhydrazine HCl	"
Wilson et al. (111)	17-hydroxy steroids	chromic acid	oxidation to 17-ketosteroids
Appleby et al. (2)(3)	17-hydroxy steroids	Na bismuthate	oxidation to 17-ketosteroids

Briefly, after the extraction of the acidified urine (pH 1.0), the steroid residue is taken up in methanol and appropriate aliquots are measured out for their assay (81). To one-half of the samples a solution of phenylhydrazine hydrochloride in diluted sulphuric acid is added, while to the remaining half of the samples, only diluted acid solution is added in order to correct for interfering substances which are chromogenic in sulphuric acid. A methanol H_2SO_4-phenylhydrazine blank is set up. The reaction is allowed to take place at 60° C. for 20 minutes and the yellow color developed is read in the Coleman spectrophotometer at 410 mμ.

The color developed is stable and the results obtained are reproducible.

In 1954, Silber and Porter (129) published a modification which permits the assay of a very few gamma of material with the dihydroxyacetone grouping.

Other modifications of this reaction are:

1. Reddy et al. (83) used the phenylhydrazine reaction on the conjugates extracted directly with butanol. Reaction was carried out in butanol.

2. The Reddy procedure has been modified by Smith (94) and Brown (14).

(a) use of laboratory purified butanol is recommended (14)

(b) adjustment of urine to pH 2.5 rather than 1.0 decreases the extraction of extraneous color (14),

(c) a longer incubation period is recommended (94).

3. The phenylhydrazine reaction has been carried out overnight at room temperature (84).

4. Nakao et al. (74) recommend the use of phenylhydrazine sulphate as the hydrochloride had been found to interfere with the spectrophotometry.

PART THREE

THE URINARY CORTICO STEROID PATTERN OBTAINED IN NORMAL SUBJECTS AND IN ADRENAL CORTICAL DISEASE

I. The Pattern in Normal Subjects

1. The Control Normal Pattern

Burton and Zaffaroni (15) (18) were the first workers to apply the technique of paper partition chromatography to the study of urinary corticosteroid patterns in normal subjects. A number of investigators including De Courcy (31), Gray (48) and Pechet (78) have done similar studies and have listed the known metabolites such as cortisone and hydrocortisone, at the same time designating by a system peculiar to their laboratory, the unidentified corticosteroids. Dr. Romanoff's group (86) studied the steroid pattern of normal, healthy subjects of both sexes from twenty to eighty-two years of age and found that many metabolites occur with enough regularity from normal individual to individual to form a fairly typical steroid pattern. In their paper there is described with partial characterization 35 metabolites.

The compounds that have been identified in the urine of normal subjects under no treatment are the following:

1. Tetrahydrohydrocortisone, THF, (6)(18)(34),
2. Tetrahydrocortisone, THE - (6) (48) (78) (92),

3. Hydrocortisone, F - (48) (15) (78) (85),
4. Cortisone, E - (15) (48) (85) (92),
5. Corticosterone, B - (85),
6. Tetrahydro-11-desoxy-17-hydroxycorticosterone, THS - (100),
7. Dihydrocortisone, DHE - (92) (85)
8. Aldosterone - (75) (105) (63)

A number of chemical and biological assays have been used to measure the various C_{21} urinary metabolites. The results obtained in normal subjects are compared in Table 4 with those observed in patients with hypofunctioning and hyperfunctioning of the adrenal cortex.

2. The Effects of ACTH Administration

Normal subjects show a significant response to exogenous ACTH stimulation. The corticosteroid levels in the blood (26)(108)and urine (42)(34) are increased.

Qualitatively the corticosteroid pattern with respect to the major urinary metabolites is essentially the same as in the untreated state and is shown in Table 5. However, Dohan et al. (34) and Touchstone et al. (102) have identified after ACTH administration the series of compounds, THB, allo-THB and THA, which have not been shown to be present in detectable amounts in the control normal urines.

The change in the corticosteroid pattern is essentially quantitative. Dohan et al. (34) published the scanning patterns of the C_{21} alpha-ketolic steroids found in normal urine before and after ACTH administration. By the third day of treatment, the pattern obtained was similar to that of

hyperfunctioning of the adrenal cortext, as in Cushing's syndrome (page 49), showing an increase when compared to the control pattern of alpha-ketolic steroid material at the positions of THE, THF, E and F.

3. The Invivo Metabolism of C_{21} Steroids

The metabolites obtained after the administration of cortisone, hydrocortisone, corticosterone and progesterone are listed in Table 6.

Also, the metabolism of dihydro E, dihydro S (103), THF and dihydro F (90) has been studied in post-menopausal women. The metabolites are listed in Table 6.

A summary of the biochemical pathways involved in the metabolism of the adrenal cortical hormones is given on page 65.

II. The Pattern in Hypofunctioning of the Adrenal Cortex and Anterior Pituitary

1. Addison's Disease

(a) Clinical Aspects

This is a primary disease of the adrenal cortex in which hormone production is sufficiently diminished to cause detectable deficiency manifestations.

In the classical form the adrenal cortex is destroyed by a local lesion such as carcinoma, haemorrhage, tuberculosis or syphilis (123, p. 143).

(b) The Corticosteroid Pattern in the Untreated State

The levels of urinary corticosteroids are significantly lower than in normal subjects and in some cases too low to be detectable. Quantitative estimations of the various C_{21} metabolites are shown in Table 4 , in comparison with the results obtained in normal subjects and in patients with other types of adrenal cortical disease.

However, Haydner (53) has studied a group of patients with one or more of the classical signs and symptoms of the disease who excrete normal quantities of 17-hydroxycorticosteroids in the urine. Paper chromatographic separation had revealed THE to be the main metabolite. (Apparently these patients are quite well normally, but when subjected to stress, they exhibit signs of adrenal cortical insufficiency.)

Qualitatively the steroid pattern is similar to that of normal subjects (30) (84) (97) (48). Burton et al. (16) found traces of THF, THE, F and E in one Addisonian but no free corticosteroids in another, and only traces of the conjugates. Insignificant amounts of material were found on the papergrams of the medium polar ($C_{21}O_4$) and least polar ($C_{21}O_3$) steroids.

Luetscher (63) has found no Na retaining hormone in the urine.

(c) The Effects of ACTH Administration

A failure to respond to ACTH stimulation, as indicated by no rise in the 17-hydroxycorticosteroid levels in plasma and urine, has been demonstrated (26) (40) (52) (108).

This is evidence that the adrenal cortical insufficiency in Addison's disease is primary and not due to lack of stimulation by endogenous ACTH secretion. In fact, the failure of the adrenals to respond to repeated doses of ACTH is regarded as a positive diagnosis for Addison's disease.

(d) The Metabolism of Administered Adrenal Cortical Hormones.

After cortisone administration a prompt increase in the excretion of tetrahydrohydrocortisone, hydrocortisone, tetrahydrocortisone and cortisone was observed in one patient by Burton (16), while no significant increase was demonstrated in the excretion of the $C_{21}O_4$ steroids. Individuals with intact adrenals were subjected to similar treatment. It was found that the increment in the excretion of the $C_{21}O_5$ steroids after cortisone administration was the same.

Gray (48) has obtained similar results with hydrocortisone treatment. The four principal metabolites in the urine accounted for about 5 to 15 per cent of the administered hormone. After giving DOC there was no increase in the levels of the most polar metabolites, suggesting that there is no conversion of the $C_{21}O_3$ to the $C_{21}O_5$ steroids.

Richardson (84) has administered compounds F, E, B, A, S, and DOC to a 55 year old male Addisonian. The metabolites obtained were similar to those obtained in a bilaterally adrenalectomized and a panhypopituitarism patient, and are tabled on page 61.

It was considered that,because of the low levels of corticosteroids in the circulation in Addisonians, there might be a state of increased tissue demand resulting in the more rapid metabolism of any administered hormones. However, Sayers et al. (89) have studied the urinary and plasma steroid levels during hydrocortisone infusion in two Addisonians, and have found the rate of metabolism to be normal.

Therefore, the biochemical pathways involved in the metabolism of the adrenal cortical hormones in Addison's disease are the same as in normal subjects. These are summarized on page 65.

2. Panhypopituitarism

(a) Clinical Aspects

Panhypopituitarism is the state in which there is "complete" failure of all anterior pituitary lobe functions including regulation of gonad, thyroid and adrenal cortical activity. Simmond's disease designates general anterior lobe deficiency (123, p. 266).

The disease is due to destruction of most or all of the anterior pituitary by local lesions such as tumors (chromophobe adenoma, craniopharyngeomas), hemorrhage, granulomas and atrophy. The condition is about four times as frequent in the female as it is in the male (123, p. 267).

(b) The Corticosteroid Pattern in the Untreated State

The levels of corticosteroids are usually subnormal. Small

amounts, 0.1 to 1.0 mgm. per day, of alpha-ketolic materials were determined by Richardson et al. (84). Other quantitative estimations of the various corticosteroids are shown in Table 4 in comparison with the results obtained in normal subjects and in patients with other diseases of the adrenal cortex. However, Gray (48) has found that,of six cases of Simmond's disease, three excreted normal amounts of corticosteroids while three had very low excretion values.

Qualitatively, the steroid patterns are similar to those obtained in normal subjects. Richardson et al. (84) have published the corticosteroid patterns of a patient with severe secondary adrenal cortical insufficiency (presumably due to a non-functioning tumor). However, the amounts of material applied to paper for chromatographic separation were too small to give peaks of alpha-ketolic material at the THF and THE positions, while no significant peaks could be observed on the papergrams of the medium polar and least polar metabolites. Alpha-ketolic material at the cortisone position was obtained on one papergram.

It has been shown that patients excrete normal levels of aldosterone or Na retaining hormone, although one of the cases had lower glucocorticoid levels (63). Venning (105) has also found that pituitary deficient patients excrete essentially normal levels of aldosterone in the urine. It would seem, therefore, as postulated by Swann (131) that the Na-regulating function of the adrenal cortex may be largely independent

of anterior pituitary control.

(c) The Effects of ACTH Administration

The results have demonstrated, in some cases, a striking response by the adrenal cortex to exogenous ACTH stimulation. Even after years of hypofunction, a response to ACTH as shown by increased glucocorticoid excretion was observed by Venning (106). Thorn (99) has found the excretion of 11-oxysteroids to be increased by seven times; Forsham (42) has found a 300 to 500 per cent increase in one patient, and Gray (48) has also observed a striking rise in the excretion of all the corticosteroids (except X_6) after ACTH administration.

Some investigators have reported subnormal responses (26) (40) (108), using the rise in plasma 17-hydroxycorticosteroids as the criterion.

(d) The Metabolism of Administered Adrenal Cortical Hormones.

Richardson et al. (84) administered separately the compounds F, E, B, A, S, and DOC to a patient.

A study of the papergrams shows that the major urinary metabolite of each compound is its tetrahydro derivative. In addition, peaks of alpha-ketolic material were found and in some cases identified in the following positions:

1. After F administration, at THE, F and E,
2. " E " " THF, F and E,
3. " B " " THA, A, allo-THB and B,
4. " A " " THB, allo-THB, B and A,
5. " DOC " " DOC

These findings are included in Table 6.

Peterson et al. (79) have shown with radioactive hydrocortisone experiments that panhypopituitarism patients can metabolize exogenous hydrocortisone at the same rate as do normal individuals.

3. Adrenalectomy

(a) Metabolic Effects of Adrenalectomy

In most animal species bilateral adrenalectomy leads to insufficiency symptoms due to the complete lack of adrenal cortical hormones. Death ensues within a few days.

In man unilateral and total bilateral adrenalectomies are carried out in order to alleviate certain pathological conditions such as Cushing's syndrome, adrenal cortical carcinoma, malignant hypertension and carcinoma of the breast (132) (96).

The fatal syndrome due to total adrenalectomy is not permitted to occur in man. High levels of adrenal cortical hormones are administered prior to and after the operation in order to prevent symptoms of acute insufficiency during surgical stress or in the post operative stage. Then, after one or two weeks time, the patients are started on a maintenance dose of cortisone and salt which they must use for the rest of their lives (132).

(b) The Metabolism of Administered Adrenal Cortical Hormones

The metabolism of the biologically active compounds, E, F, B, A, S, and DOC has been studied in an adrenalectomized, orchidectomized male, 65 years of age (84). The metabolic pathways are similar to those observed in an Addisonian and a panhypopituitarism patient described previously. The compounds found are listed in Table 6.

The fate of tracer doses of hydrocortisone-4-C^{14} given intravenously to an adrenalectomized, oophorectomized patient has been studied by Hellman et al. (55) when,

1. All replacement therapy was withdrawn,
2. Large doses of adrenal cortical hormones were administered.

No difference in the metabolism of the radioactive hydrocortisone was demonstrated under these physiological conditions. These results substantiate those obtained by Sayers after the infusion of hydrocortisone to two patients with Addison's disease. Apparently, the existing levels of the circulating adrenal cortical hormones do not affect the rate of metabolism of these compounds after their administration by intravenous technique.

No Na retaining substance was excreted in the urine of two adrenalectomized patients studied by Luetscher (63). This is evidence that aldosterone is produced by the adrenal glands.

III. The Pattern in Hyperfunctioning of the Adrenal Cortex and Anterior Pituitary

1. Cushing's Syndrome

(a) Clinical Aspects

Cushing's disease may be due primarily to dysfunction of the anterior pituitary basophilic cells. There is as a secondary effect a hyperplasia or hyperactivity of the adrenal cortical glands. A primary defect of the adrenal cortex, due to a tumor, may also be a cause.

(b) Corticosteroid Patterns in Typical Cushing's disease.

Increased amounts of the adrenal cortical hormones and their metabolites in the urine have been demonstrated by Anderson (cortin) (1), Weil and Browne (cortin) (109), and Wilson (Porter-Silber chromogens) (112). The quantitative estimations obtained with other chemical and biological assays are shown in Table 4. In 1948, Mason (71) first isolated hydrocortisone in large amounts (14 to 19 mgm. per day) from the urine of a 14 year old boy with the disease. The levels of Na-retaining hormone excreted in the urine are elevated (75) (76) (105).

However, some patients with no significant irregularities in carbohydrate metabolism may excrete essentially normal levels of corticosteroids (30). Lunnon (64) has found very low 17-ketogenic values in some individuals.

Smith et al. (95) have studied the urinary corticoid

excretion during the day and night and have found the excretion rates to be similar. This is in contrast to the results obtained with normal subjects by Pincus (80) and Migeon (72). In the normals there are lower excretion values in the night.

The urinary corticosteroid patterns have been studied by a number of investigators, including Gray (48), Wilson (112), and Dohan et al.

The following patients were studied by Dohan's group (34):

1. A 19 year male with florid Cushing's Syndrome with no evidence of a tumor,
2. A 45 year female with increasing hirsutism, diabetes mellitus and severe hypertension (autopsy revealed hyperplasia and an increase in pituitary basophils),
3. A 45 year female with moderate hirsutism and increased corticosteroid and 17-ketosteroid levels.

A comparison of the chromatographic patterns of these three cases revealed a general similarity as well as with those obtained in normal individuals after three days treatment with ACTH.

The compounds THE, THF, F and E were identified. A large peak at the F position was present in all three Cushing's syndrome cases, also at the THF, THE and E positions. In the florid Cushing's syndrome case 60 per cent of the steroids present in the E position on the papergram was due to THS.

According to the authors (34), in this disease there is evidence for a change in adrenal cortical secretion. The proportion of the most polar steroids is 83 to 88 per cent of the total while in the normal it is 57 to 71 per cent of the total. In normal subjects on one day of ACTH treatment the proportion of highly polar corticosteroids is 50 to 63 per cent, whereas after three days of ACTH treatment it is 86 per cent - i.e., similar to the diseased state.

These studies revealed a noticeable increase above normal of compounds B and A in only one of the three cases presented. Bush has found that in the adrenal venous blood of a Cushing's syndrome patient, the steroids F and B were in the ratio of 20 to 1. This is evidence that prolonged stimulation by ACTH causes a shift in adrenal cortical secretion so that F and E are increased in comparison to compounds B and A. Also a study of the papergrams shows peaks of unidentified alpha-ketolic compounds in positions corresponding to those of a series of three compounds detected in the patterns of normal subjects after ACTH treatment. These compounds were identified to be THB, allo-THB and THA.

Therefore, the work of these investigators and of others suggests that the clinical and biochemical manifestations of the disease are due to prolonged stimulation by abnormally high levels of ACTH (35). Measurements of blood levels of ACTH have given varied results, but Rubin et al. have found increased levels of ACTH in Cushing's syndrome urine (34).

It has also been reported that hypophysectomy has led to a remission in one case (35).

However, as Dorfman (35) points out, hydrocortisone normally inhibits ACTH secretion and yet in this disease above normal levels of ACTH are secreted in the presence of high circulating levels of hydrocortisone. The author suggests that there is a defect in the anterior pituitary which causes it to be insensitive to the levels of adrenal cortical hormones and to continue to pour out ACTH which in turn overstimulates the adrenals.

(c) Corticosteroid Pattern in Adrenal Cortical Carcinoma

Dohan et al. (34) have studied a 48 year old female with a mixture of the Cushing and adrenogenital syndromes, due to adrenal cortical carcinoma. She had marked hirsutism and abnormalities of electrolyte metabolism. The 17-keto-steroid levels were 162 to 320 mgm. per day while the normal values are about 10 mgm., and the reducing lipid determination was 64 mgm. per day - the normal value is about 20 mgms.

Previous work (101) on this patient had revealed the presence of THS in the urine. The papergrams (34) indicated that THS was by far the major urinary metabolite. The presence of only small levels of THF and THE was indicated, whereas in normal subjects and in the three Cushing's syndrome cases previously described, these were the major metabolites.

The small peak of alpha-ketolic material on the paper

chromatogram of the medium polar compounds was presumed to be compound S, although it was not conclusively proved to be so. (Previous work on the metabolism of compound S demonstrated that 90 per cent of the alpha-ketolic material excreted was its tetrahydro metabolite, THS (84).

Apparently, the major adrenal cortical hormone produced by this adrenal cortical carcinoma tissue was compound S which has only a weak suppressing effect on ACTH secretion. Therefore, enough ACTH was put out to stimulate the remaining normal tissue to secrete some hydrocortisone and cortisone, resulting in the appearance of their reduced metabolites in the urine (34).

(d) The Response to the ACTH Test

The type or degree of response obtained during ACTH administration depends upon the cause of the hyperfunctioning.

1. Cushing's syndrome patients with bilateral hyperplasia of the adrenals show an excessive response with respect to increased corticosteroid excretion (59) (26) (108) (40) (52).

2. If the disease is due to a benign tumor or adenoma, the extent of the response may depend upon the degree of autonomy or independence of the tumor with respect to anterior pituitary control (59).

3. In adrenal cortical carcinoma there is no increase in plasma or urinary levels of corticosteroids (59) (40). However, the levels of 17-ketosteroids are markedly increased (35).

2. Adrenogenital Syndrome

(a) Clinical Aspects of the Various Types

The adrenogenital syndrome is due to hyperactivity of the adrenal cortex. It has been differentiated into more than one disease.

(i) The type acquired after birth is usually due to an adrenal cortical tumor (46) and is a mixture in varying proportions of the adrenogenital and Cushing syndromes. There is excessive production of androgens and marked body hirsutism. The levels of corticosteroids in the urine are not necessarily elevated (1) (109). There is no response to cortisone administration (110).

(ii) In the congenital variety there is bilateral hyperplasia of the adrenals. Patients suffering from congenital adrenal or virilizing, hyperplasia can be divided into 3 main groups (110):

1. Those with symptoms of virilization only,
2. Those with the Na losing syndrome as well,
3. Those with hypertension as well.

(b) The Urinary Corticosteroid Pattern in Congenital Adrenal-hyperplasia

(i) The Results of Chemical Assays

The studies on blood have revealed low corticosteroid levels (11) (12) (110); but most standard chemical methods have failed to show low steroid levels in the urine. Porter-

Silber chromogenic, α-ketolic, formaldehydogenic steroids and 11-oxysteroid determinations gave essentially the same results in normal individuals and in patients, while the dinitrophenylhydrazine assay was somewhat lower in patients. However, most significantly, the acetaldehydogenic steroids were markedly elevated - about 10 times the normal value (37) (110).

Also, the levels of the neutral 17-ketosteroids and the 17-ketogenic steroids were significantly increased (110).

(ii) The nature of the C_{21} metabolites present in the urine

In order to determine which compounds were actually measured by these assays, Eberlein et al (37) used paper chromatographic separation. The free and ensyme hydrolysed fractions of 24 hour urines were separately studied using chromatographic patterns of normal subjects as controls and Cushing's syndrome patterns for comparison.

Their papergrams showed no detectable F or E in the free fraction, while in the enzyme hydrolysed fraction only a small amount of THE was found. None of these compounds was found in 30 litres of urine from one of the patients. These results were a striking contrast to those obtained in Cushing's syndrome. When the papergram findings were taken into consideration, it was obvious that E, F and their reduced metabolites were not to any extent the compounds measured by the Porter-Silber reactions, but other unidentified 21-hydroxylated compounds present in the urine.

Brooks, Bongiovanni and others (12) (69) found pregnantriol to be regularly occurring in very much increased amounts in the urine of patients. Pregnantriol-11-one and pregnandiol have also been found but in smaller amounts. These compounds could account for the excessive 17-ketogenic and acetaldehydogenic steroid values.

Chromatographic separation (column) showed that the quantities of 11-oxygenated-17-ketosteroids were disproportionately increased and accounted for 18 to 25 per cent of the total neutral 17-ketosteroids,while in normal subjects the percentage is 10 (37).

(iii) The interpretation of the steroid pattern

Since the most characteristic findings were increased levels of C_{21}-desoxy compounds (pregnantriol, pregnantriol-11-one and pregnandiol), the authors postulated that there is a block due to an enzyme deficiency at one or more points (C11,17,21) in the synthesis of hydrocortisone (12). However, Dorfman (35) considers the main defect to be a lack of C_{21}-hydroxylating enzymes.

Hechter had done some work on adrenal perfusions which demonstrated that ACTH stimulates the synthesis of progesterone via pregnenolone from cholesterol. The progesterone is then successively hydroxylated at carbons 11, 17 and 21 to form hydrocortisone.

Wilkins et al. (110) suggest that there might be a defect

in the metabolism of 17-hydroxyprogesterone (110), a precursor of hydrocortisone which would result in,(1) very low F secretion, (2) high pregnantriol values, and (3) excessive 17-ketogenic values.

Jailer et al. (57) gave exogenous 17-hydroxyprogesterone and 21-desoxyhydrocortisone to patients with adrenocortical insufficiency so that the endogenous steroid levels were low. (The progesterone derivative was of interest as it had been previously isolated from extracts of adrenal cortical tissue and had been shown to have androgenic activity. Although 21-desoxy F had not yet been studied, 21-desoxy E had been reported to be metabolized to 21-desoxy THE and to 11-oxygenated-17-ketosteroids.)

The urinary steroid patterns of these patients were then compared to those obtained in patients with untreated congenital adrenal hyperplasia in order to see whether the two administered compounds could account for the C_{21} and C_{17} metabolites found in the disease. A general similarity was observed.

Consequently, Jailer suggests that the androgenic manifestations and the high 17-ketosteroid levels may be due to increased 17-OH progesterone (110) elaboration. However, Bongiovanni has not been able to find increased levels of this compound in the blood or urine (110) of patients. Dorfman considers the androgenic properties to be too weak to account for the virilizing symptoms.

Morris (35) considers the primary defect in congenital adrenal hyperplasia to be excessive C_{19} steroid production by the adrenal cortex due to selective stimulation by high ACTH levels.

(c) Atypical Steroid Pattern in a Case with Hypertension

The urinary corticosteroid pattern was studied in such a case and was shown to be very different from those previously recorded in patients without hypertension (36).

The patient was an $8\frac{1}{2}$ year old hermaphrodite with exceptionally high plasma levels of 17-hydroxycorticosteroids.

The free urinary corticosteroids were separately chromatographed on paper. The single steroid obtained had the mobility of compound S. The enzyme hydrolysed fraction was also paper chromatographed and the predominant steroid was identified to be tetrahydro-S. It was excreted at the rate of 18 mgm. daily and, according to the authors, the only previous case showing such a high urinary content of THS was that of adrenal cortical carcinoma (101). A second steroid isolated was tentatively identified as tetrahydrodesoxycorticosterone (THDOC) and, although THDOC was previously identified in the urine after the administration of DOC (84), this was the first time that it was found to occur in urine from endogenous sources. Pregnantriol, pregnandiol and pregnane-3,α 17α, 20?, 21-tetrol were isolated. No traces of F or its metabolites were detected.

Significantly, all the compounds present in the urine were shown to have no oxygen function at C_{11}. Similarly, investigation of the 17-ketosteroids revealed the absence of 11-oxygenated-17-ketosteroids.

During cortisone therapy this unusual steroid pattern disappeared together with the lessening of hypertension.

The authors (36) suggest that this case of congenital adrenal hyperplasia was due to a deficiency of the adrenal enzyme or enzymes necessary for C_{11} hydroxylation. This deficiency would result in the piling up of such 11-desoxy compounds as S and DOC, while in turn the resulting DOC secretion might be the cause of the severe hypertension.

(d) The Response to the ACTH Test

Early it was found that the response to ACTH was unusual. There were the following laboratory observations:

(i) No appreciable rise in urinary corticosteroid levels (7) (11) (12).

(ii) No rise in the already low levels of blood corticoids (26) (52) (108). (However, some investigators have observed a response in some cases.) (12) (32) (110).

(iii) A still further increase in the already elevated amounts of urinary 17-ketosteroids (32).

(iv) A disproportionate rise in 11-oxygenated-17-ketosteroids (32).

(v) A significant increase in pregnantriol excretion (32) (110).

(e) The Response to Cortisone Therapy

Wilkins first showed that cortisone treatment arrests all the clinical manifestations of the disease. The urinary corticosteroid and C_{19} steroid pattern returns to normal with:

(i) A fall or disappearance of pregnantriol excretion in the urine.

(ii) A suppression of 17-ketosteroid output with the restoration of the normal proportion of 11-oxygenated compounds (7) (59).

(iii) The presence of F-like compounds in the blood and urine due to the metabolism of cortisone.

(iv) A decrease in the oestrogen levels. Oestrogens are increased in the disease but they exert no peripheral effect because of the greater excess of androgens in the circulation (110).

QUANTITATIVE ESTIMATIONS IN NORMAL SUBJECTS AND IN PATIENTS WITH ADRENOCORTICAL DISEASE

Material Measured	Normal	Hypofunctioning	Hyperfunctioning	References
glucocorticoids units/24 hr.	25-55 (f.) 40-85 (m.)	0 - 25 (Addison's) 0 - 25 (hypopit.)	40-500 (Cushing's) 700 "	Venning et al. (106)
formaldehydogenic steroids mgm./24 hr.	1.0-1.6	0.3-0.65 (Addison's) 0.5-0.8 (hypopit.)	23 (Cushing's) 1.8-2.2 "	Daughaday et al. (30)
11-oxycorticosteroids mgm./24 hr.	0.1-4.4	0.02-0.29 (Addison's) 0.010 (panhypopit.)	1.45 (adr. neoplasm) 0.6-12.0 (Cushing's)	Talbot et al. (97)
tetrahydrocortisone mgm./24 hr.	2.0-7.4 (m.) 3.3 (f.)	0.4 (panhypopit.)	12.1 (Cushing's)	Baggett et al. (60)
tetrahydro-S γ/24 hr.	20 (pooled urine)		1700 (Cushing's) 15,000 (adr. neoplasm)	Touchstone et al. (100)
11-oxysteroids mgm./24 hr.	0.75-2.0		3.2-11.3 (Cushing's)	Soffer et al. (96)

TABLE 4

TABLE 5

C_{21} STEROIDS ISOLATED FROM URINE AFTER ACTH ADMINISTRATION

Type of Subject	Urinary C_{21} Steroids Isolated	Reference
Normal	pregnane-3α,11β,17α,21-tetrol-20-one (THF) pregnane-3α,17α,21-triol-11,20-dione (THE) pregnane-3α,11β,21-triol-20-one (THB) allopregnane-3α,11β,21-triol-20-one (allo-THB) pregnane-3α,21-diol-11,20-dione (THA) corticosterone (B) hydrocortisone (F) cortisone (E)	Dohan et al. (34)
Hodgkins disease	pregnane-3α,11β,17α,20α,21-pentol pregnane-3α,17α,20α,21-tetrol-11-one pregnane-3α,11β,17α,20β,21-pentol pregnane-3α,17α,20β,21-tetrol-11-one	Fukushima et al. (43)
Normal	pregnane-3α,11β,21-triol-20-one (THB) allopregnane-3α,11β,21-triol-20-one (allo-THB) pregnane-3α,21-diol-11,20-dione (THA) corticosterone (B)	Touchstone et al. (102)
Normal	hydrocortisone pregnane-3α,17α,21-triol-11,20-dione pregnane-3α-ol-20-one	Lieberman et al. (61)
Normal	hydrocortisone (F) pregnane-3α,17α,21-triol-11,20-dione (THE) pregnane-3α-ol-20-one	Lieberman et al. (61)
Normal	pregnane-3α,17α,21-triol-11,20-dione (THE) pregnane-3α,11β,17α,21-tetrol-20-one (THF) hydrocortisone cortisone	Gold et al. (45)

THE IN VIVO METABOLISM OF C_{21} STEROIDS TO C_{21} METABOLITES IN HUMAN SUBJECTS

Steroid Administered	Type of Subject	C_{21} Steroids Isolated From Urine	References
E	normal male	pregnane-3α,11β,17α,21-tetrol-20-one pregnane-3α,17α,21-triol-11,20-dione hydrocortisone	Burstein et al. (20)
F	scleroderma, m.	pregnane-3α,11β,17α,21-tetrol-20-one pregnane-3α,17α,21-triol-11,20-dione cortisone	Burstein et al. (19)
21-desoxy E	rheumatoid arthritis, f.	pregnane-3α,17α-diol-11,20-dione	Burstein et al. (120 p. 138)
DHE	post menopausal woman (2)	pregnane-3α,17α,20α-triol pregnane-3α,17α,21-triol-20-one no allopregnane derivatives	Ungar et al. (103)
B	rheumatoid arthritis	allopregnane-3α,11β,21-triol-20-one pregnane-3α,11β,21-triol-20-one pregnane-3α,21-diol-11,20-dione pregnane-3α,20α-diol-11-one	Engel et al. (38)
B	rheumatoid arthritis, m.	allopregnane-3α,11β,21-triol-20-one pregnane-3α,21-diol-11,20-dione	Engel et al. (39)
B	normal	pregnane-3α,11β-diol-20-one pregnane-3α,11β,20α-triol-3,20-dione	Kemp et al. (58)

TABLE 6

THE IN VIVO METABOLISM OF C_{21} STEROIDS TO C_{21} METABOLITES IN HUMAN SUBJECTS (continued)

Steroid Administered	Type of Subject	C_{21} Steroids Isolated From Urine	References
F	normal, hypoadrenalism, rheumatoid arthritis	hydrocortisone 20β-hydroxyhydrocortisone 6β-hydroxyhydrocortisone pregnane-3α,11β,17α,21-tetrol-20-one (THF)	Peterson et al. (79)
THF DHF	postmenopausal women (2)	pregnane-3α,17α,21-triol-11,20-dione (THE) pregnane-3α,11β,17α,21-tetrol-20-one (THF)	Savard et al. (90)
17-hydroxy-progesterone	hypoadrenalism (2) pseudohermaphrodite	pregnane-3α-ol-20-one pregnane-17-ol-3-one pregnane-3α-ol-11-one	Jailer et al (57)
21-desoxy F	hypoadrenalism hypothyroidism	pregnane-3α,11β,17α-triol-20-one (21-desoxy THF) pregnane-3α,17α-diol-11,20-dione (21-desoxy THE)	Jailer et al. (57)
E and ACTH	normal	pregnane-3α,17α-diol-11,20-dione (21-desoxy THE) Δ^5-pregnen-3β,21-diol-20-one	Lieberman et al.(60)
F	hypoadrenalism (2) adrenalectomized (1)	pregnane-3α,11β,17α,21-tetrol-20-one, THF cortisone, E pregnane-3α,17α,21-triol-11,20-dione, THE	Richardson et al. (84)
E	"	same as above, dihydrocortisone	

TABLE 6

THE IN VIVO METABOLISM OF C_{21} STEROIDS TO C_{21} METABOLITES IN HUMAN SUBJECTS (continued)

Steroid Administered	Type of Subject	C_{21} Steroids Isolated From Urine	References
A	hypoadrenalism (2) adrenalectomized (1)	pregnane-3α,21-diol-11,20-dione,THA pregnane-3α,11β,21-triol-20-one,THB corticosterone	Richardson et al. (84)
B	"	same as above also allopregnane-3α,11β,21-triol-20-one, allo-THB	
S	"	pregnane-3α,17α,21-triol-20-one,THS	
DOC	"	pregnane-3α,21-diol-20-one,THDOC	

TABLE 6

SUMMARY OF THE METABOLISM OF ADRENAL CORTICAL HORMONES IN HUMANS

1. Small amounts of biologically active corticosteroids are excreted in an unaltered form. These include compounds F, E, B and aldosterone.

2. Little is known of the metabolic pathways involving the degradation of the cyclopentanoperhydrophenanthrene nucleus. Hellman et al. (55) studied the in vivo metabolism of hydrocortisone-C^{14}. They regard their failure to find radioactivity in the expired carbon dioxide as an indication that there is no breakdown of the steroid nucleus.

3. Reduction of the corticosteroid molecule for biological inactivation and excretion (after conjugation) is a major metabolic pathway.

   (a) The main metabolites are the tetrahydro derivatives of the pregnane (5β) series.

   (b) Only after the administration of progesterone and corticosterone have smaller amounts of the tetrahydro allopregnane (5α) series been found.

   (c) Small amounts of the partially reduced, dihydro derivatives, are excreted after the administration of cortisone (84), and in normal urine (92).

4. Small amounts of corticosteroids, those with a 17 -hydroxy-20-ketone grouping, are converted to 17-ketosteroids by oxidative degradation of the C_{17} side chain (20) (19) (57).

With few exceptions, e.g. congenital adrenal hyperplasia, the urinary-corticosteroid patterns differ only quantitatively in normal subjects and in adrenocortical and anterior pituitary disease.

E X P E R I M E N T A L

I. Scope of the Investigation

As mentioned in the introduction, the purpose of this investigation was to study the urinary corticosteroid pattern in adrenal cortical and anterior pituitary disease, by the technique of paper chromatography.

Specifically, the scope of this work was to obtain and study the urinary corticosteroid pattern of the metabolites ranging in polarity from THE to A (in the Zaffaroni toluene-propylene glycol system) in 2 normal subjects, 2 cases of Cushing's syndrome, 1 case of congenital adrenal hyperplasia and in 3 cases of hypopituitarism.

An attempt was made to obtain the corticosteroid pattern of each patient under various physiological conditions. Hence, urine samples were collected while,(a) the patient was in the active, untreated disease state, and (b) while the patient was submitted to an ACTH test or cortisone therapy, and in 2 cases to surgical treatment as well.

The urine samples were then submitted to the following procedure:

1. The corticosteroids were extracted.

2. The crude urinary extracts were then submitted to fractionation by the technique of paper chromatography, using the Zaffaroni systems of benzene-formamide and toluene-propylene glycol. Although the chromatographic procedures for the different cases varied to some extent, an attempt was made to chromatograph the various extracts in such a way that the urinary corticosteroid patterns could be easily compared. This was to facilitate the evaluation of any qualitative and quantitative differences within the different sets of patterns of (a) a single patient and (b) of the normal subjects and various disease states.

3. The chromatograms were then 'scanned' in such a way that a picture, or pattern, of the sequence of the metabolites with either the α,β-unsaturated grouping, or the primary alpha-ketol grouping, or both, was obtained for each extract.

These scanning patterns are the so-called urinary corticosteroid patterns referred to in this study and are essentially the results of this study.

4. Then, using the scanning patterns as a guide, various zones of the paper chromatograms were eluted and subjected to further characterization studies, and in some cases to quantitative estimation.

It must be made clear at this point that no metabolite was absolutely identified since no infra-red spectra were obtained. However, it seemed most logical to designate certain

regularly occurring metabolites to be the compounds THF, THE, F and E, if these had the same chromatographic, other physical, and chemical behaviour as the standards are known to have. On the other hand, other regularly occurring metabolites were referred to as X_2, X_4, X_8, etc., and an attempt was made to characterize some of them.

II. The Methods

A. Hydrolysis and Extraction

1. Reagents

Chloroform - c.p., distilled prior to use.

Beta-glucuronidase - "Ketodase" from the Warner-Chilcott laboratories, a beef liver preparation with a potency of 5,000 units per ml. of solution.

Buffer tablets - Coleman, pH 5.0.

1/10 N NaOH solution.

2. Technique

All the urine samples studied were processed in an identical manner. A 12 hour aliquot or more from a 1 or 2 day urine collection was extracted three times. The procedure is outlined on a flow sheet on the following page. The urine was first extracted at about neutral pH in order to obtain the free fraction, A, and then re-extracted after 24 hours of enzyme hydrolysis in order to obtain the fraction conjugated with glucuronic acid, B. The third extraction, C, was carried out after a further 24 hours of enzyme hydrolysis and immediately after the acidification of the urine to pH 1.0. Extract C contained more liberated glucuronides and also any steroids that might be present as conjugates that are readily hydrolysed by acid, such as sulphates.

The combined extracts A, B, and C were washed with alkali in order to remove most of the extraneous urinary pigments

Procedure for the hydrolysis and extraction of urinary corticosteroids

Urine - p.H. 6.0
extracted with ½, ¼, ¼ vols. $CHCl_3$

$CHCl_3$ extract A
evaporated down in vacuo below 50°C.

Urine A.
1. acidified to pH 5.0 with conc. H_2SO_4
2. added 1 crushed buffer tablet per litre of urine
3. added 200 units of B-glucuronidase per ml. of urine
4. incubated at 37°C for 24 hours
5. extracted with ½, ¼, ¼ vols. $CHCl_3$

$CHCl_3$ extract B
evaporated down in vacuo below 50°C, combined with A

Urine B.
1. checked for pH 5.0
2. incubated for a further 24 hours
3. acidified to about pH 1.0
4. extracted with ½, ¼, ¼ vols. $CHCl_3$

$CHCl_3$ extract C
combined with extracts A and B
evaporated down to 400 c.c. (if necessary)
washed with 5 x 40 c.c. of cold 0.1N $NaOH$ solution
washed with 2 x 40 c.c. of distilled water
evaporated to near dryness in vacuo, below 50°c., dried under Nitrogen

Urine C.
discarded

and the acidic and phenolic steroid fractions. The neutral fraction remained in the chloroform. After the removal of the solvent the dried steroid residue was ready to be applied to paper.

B. Paper Chromatography

1. The Materials

The chromatographic jars, other chromatographic equipment and solvents which have been used were those as described by Zaffaroni (17).

2. The Technique

The filter paper used for chromatography was washed with methanol for 2 days in a Soxhlet extractor and air dried prior to use. The length used was the standard 42 cm. and the starting line was always drawn 12 cm. away from the upper edge. The width of paper used varied depending upon the amount of material that was applied.

After the paper was impregnated with the appropriate stationary phase, it was blotted a few times between sheets of filter paper. The terms undiluted and diluted, written in the text after the solvent system used, have the following significance. The former is used to indicate that stationary phase (either formamide or toluene propylene glycol) was used alone for impregnation. The latter term is used to indicate that the stationary phase was diluted with an equal part of methanol in order to increase the running rates of the corticosteroids applied.

The application was carried out as follows. A few drops of chloroform-methanol (1:1) were added to the dried steroid residue in a small test tube. Then the solution was applied quantitatively to the starting line by means of a micropipette. After each application the starting line area was dried by a stream of air.

The papers were then placed in the chromatographic chambers and development was carried out either at room temperature or at 31° C. in a constant temperature cabinet. The running time necessary for adequate separation of the corticosteroids varied from jar to jar and especially with the material applied. It was found that the pure standards which were applied on separate strips of every chromatogram usually ran significantly faster than did the same compounds present in the urine extracts. This was due to the presence of impurities in the latter which, depending upon their amount, caused a retardation of the flow of the mobile phase.

3. The Benzene-formamide System

The most important use of the benzene-formamide system was for the further purification of the crude, alkali washed, extracts and for the rough fractionation of the steroid material, as described by Burton *et al.* (17) Chromatography was carried out at room temperature and the following fractionation was obtained:

(i) The 0 to 5 hour overflow was collected separately. It contained most of the extraneous urinary pigment and fatty material and the $C_{21}O_3$ corticosteroids, such as DOC, if any were applied on the paper. This fraction was not used in any further chromatographic studies.

(ii) The 5 to 48 hour overflow was collected separately. This fraction contained the less polar, $C_{21}O_4$, corticosteroids such as compounds B and S, and was rechromatographed on the 24 hour T-PG papergram, as described below.

(iii) The most polar, $C_{21}O_5$, corticosteroids, mainly THF, THE, F and E were retained on the paper at a distance ranging from 0 to approximately 11 cm. away from the starting line.

The remainder of the B-F papergram, i.e. from about 11 cm. to the end of the paper, retained, to some extent, the more polar $C_{21}O_4$ metabolites such as the reduced tetrahydro derivatives of compounds S, B and A. In the faster running papers, parts of this fraction were also found in the 5 to 48 hour overflow.

4. The Toluene-propylene Glycol System

The toluene-propylene glycol system was used to obtain the final corticosteroid patterns shown in the section on results.

The various standard compounds that have been separated in the T-PG system are listed as follows in an order of decreasing polarity:

1.	THF:	$C_{21}O_5$	-	more polar
2.	THE:	"	-	" "
3.	F:	"	-	less polar
4.	E:	"	-	" "
5.	THS:	$C_{21}O_4$	-	more polar
6.	THB:	"	-	" "
7.	THA:	"	-	" "
8.	S:	"	-	less polar
9.	B:	"	-	" "
10.	A:	"	-	" "

The most regularly occurring urinary metabolites such as THF, THE, F, E, and B can be separated to some extent over the length of two T-PG papergrams. However, in an effort to achieve a clearer resolution, the corticosteroids ranging in polarity from that of THF to that of compound A were separated, in most cases, over a series of three chromatograms in this study.

Consequently, an attempt was made to rechromatograph the B-F papergrams with their respective 5 to 48 hour overflows with the following aim:

(i) To include on the 6 to 8 day T-PG papergram the pattern of the more polar $C_{21}O_5$ metabolites ranging in polarity from that of THF to that of F, for all cases studied.

(ii) To include the pattern of the less polar $C_{21}O_5$ steroids, such as E, and part of the pattern of the more polar $C_{21}O_4$ steroids, such as THS, on the 2 to 3 day T-PG papergrams. This was attempted for all cases except A.L. and D.M. - here no $C_{21}O_4$ steroids were applied on the 3 day paper, i.e. the lower part of the B-F papergrams were not rechromatographed.

(iii) To include the pattern of the more and less polar $C_{21}O_4$ metabolites ranging in polarity from THB to A on the 24 hour T-PG, undiluted papergram, for all cases studied.

The details of the chromatographic procedure used for each case are described below.

The following method was used for the normal subjects and for case S.K.

(i) An aliquot of the eluate of the starting line area of the initial B-F papergram was rechromatographed for 7 days in T-PG, diluted.

(ii) An aliquot of the eluate of the entire B-F papergram was rechromatographed for 3 days in T-PG, diluted.

(iii) An aliquot of the lower part of the B-F papergram was combined with an equal concentration of the 5 to 48 hour overflow and was rechromatographed for 24 hours in T-PG, undiluted.

The following method was used for cases A.L. and D.M.

(i) An aliquot of the eluate of the starting line area of the initial B-F papergram was rechromatographed for 7 days in T-PG, diluted, while another aliquot was rechromatographed for 3 days in T-PG, diluted.

(ii) An aliquot of the lower part of the B-F papergram was combined with an equal concentration of the 5 to 48 hour overflow and was rechromatographed for 24 hours in T-PG, undiluted.

The following methods were used for cases G.A. and F.T.

A single B-F purification run was sufficient for all the extracts except for those of case G.A. This case was the only one not extracted during the course of the investigation, but about a year earlier. The extracts originally contained much extraneous pigment and fatty material and, after standing for many months in the refrigerator, they became jelly-like in consistency and rather difficult to apply on paper. As a result, the 'preliminary chromatography' was of necessity more extensive. A second purification run, using the T-PG system, was carried out for extracts (a) and (b). Then each of the three extracts was chromatographed separately in T-PG for 5 days, 2 days and 24 hours, respectively. However, for reasons described below equal concentrations were removed from the eluates of each of these 3 T-PG papergrams for every extract and were re-chromatographed on separate strips of one paper in T-PG, diluted, for 7 days. The overflow from this paper was rechromatographed in T-PG, diluted for 3 days while the overflow from this latter paper was rechromatographed in T-PG, undiluted, for 24 hours.

The crude extracts of case F.T. were relatively clean. Thus an aliquot of the eluate of the entire B-F papergram was combined with an equal concentration of the 5 to 48 hour overflow and rechromatographed for 8 days in T-PG, diluted. The overflow from this paper was rechromatographed for 3 days in T-PG, diluted while the overflow from this latter paper was rechromatographed for 24 hours in T-PG, undiluted.

5. The Four-jar System

This method was used only for case W.R. The extracts of the free and enzyme hydrolysed fractions were chromatographed on separate strips of one paper using the following technique of Zaffaroni (114).

The crude alkali washed extracts were applied on formamide impregnated paper and developed by transferring from jar to jar in a series of 4 jars containing n-hexane, n-hexane-benzene (1:1), benzene and chloroform, respectively. A four hour overflow was collected in each jar and only the $C_{21}O_5$ steroids were retained on the paper developed in the chloroform chamber. This paper was then entirely eluted and the eluate was rechromatographed in the T-PG, diluted system for 48 hours.

Whenever it was possible, it was attempted to chromatograph the various extracts of a single patient on separate strips of the same T-PG papergrams. Thus, in order to make any difference in the 2 or 3 sets of corticosteroid patterns more apparent, and to facilitate visual comparison (of the scanning patterns), the chromatographic conditions (jar, temperature, time) were made identical for each of the extracts.

6. The Concentrations Applied

The concentration of material applied to the paper is expressed as hours of urine aliquot per cm. width of strip, i.e. 0.8 hours per cm. means that an amount of corticosteroid material, equivalent to 0.8 hours volume of a 24 hour collection, was applied for every cm. width of strip.

In order to facilitate visual comparison, it would have been best to chromatograph the same concentration of material for all the cases studied. Thus 0.8 hours per cm. was applied on all the B-F papergrams and on the T-PG papergrams of the normal subjects and cases S.K. and G.A. However, it was thought better to use a greater concentration for cases where the levels of glucocorticoids, such as F and E, were expected to be very low. Consequently, 1.4, 1.6 and 1.9 hours per cm. were used for patients A.L., F.T. and D.M., respectively. The concentration used for case W.R.was 1.2 and 6.0 hours per cm. for the enzyme hydrolysed and free fractions, respectively.

C. Detection and Scanning of Corticosteroids on the Chromatograms

The next step in this investigation was to establish the positions of the individual corticosteroids separated upon the series of chromatograms, on the basis of their behaviour

in the spotting tests and scanning procedures, described below. The UV absorption test detected the alpha, beta-unsaturated steroids and the BT reaction, the primary alpha-ketolic material.

1. Reagents

(a) Blue tetrazolium (BT):

(i) The BT solution for the spot test consisted of 0.25 cc. of 2% aqueous BT solution, 3.75 cc. of 10% aqueous NaOH solution and 1 cc. of distilled water.

(ii) The BT solution used for scanning consisted of 25 cc. of 2% aqueous BT solution, 37.5 cc. of 60% aqueous methanol and 37.5 cc. of 10% aqueous NaOH solution.

Both solutions were prepared freshly prior to use.

(b) 2N H_2SO_4 solution.

2. Techniques

(a) Spot Tests

(i) Ultra violet light absorption (UV)

The dried paper chromatogram was studied at 240 mμ, with the aid of a mercury hand lamp. The areas of UV absorption were marked and a rough visual estimation of the degree of absorption, ranging from negative to four plus, was made and recorded. This was done for every chromatogram developed.

Some of the chromatograms were exposed for a few seconds to UV light in the region of 240 mμ in a print box, and photo-prints of compounds with the Δ^4-3-ketone grouping were obtained, providing an objective and permanent record.

(ii) Blue tetrazolium reduction (BT)

The solution, (a)(i), was applied on a 0.2 to 0.5 cm. wide strip of paper cut from the entire length of the chromatogram. The presence of BT reducing compounds was indicated by blue to purple staining areas. The positions of these areas were recorded. This test was used mainly to establish the positions of the standards and unknown compounds, with reduction at ring A.

(b) Scanning Patterns

(i) Ultra violet light absorption

A one cm. strip of the entire length of the chromatogram was then removed and scanned at 240 mμ in a "Photovolt" photoelectric densitometer in such a way that readings of the optical densities were measured from three cm. above the starting line to the end of the strip. They were then plotted against distance from the starting line, and a pattern of peaks of UV absorbing compounds was thus obtained, showing the Δ^4-3-ketonic material.

(ii) Blue tetrazolium

The strips of paper that were scanned at 240 mμ were reacted with BT by:

1. immersing the strip in the basic BT solution, (a)(ii), for anywhere from a few seconds to three minutes time - the greater the concentration, the less was the time

required for maximum color development,

2. dipping the strip in 2N H_2SO_4 solution in order to stop the reaction,

3. washing in distilled water in order to remove excess reagent.

The strips were then dried in an oven for a few minutes at 80° C.

The paper strip was then scanned in the photoelectric densitometer at 540 mμ in an identical manner. The optical densities of the BT reducing compounds were thus recorded and plotted so that the pattern of the peaks of compounds with the primary alpha-ketol grouping was obtained.

The plotting was done in such a way that both types of patterns were superimposed, resulting in the formation of the so-called urinary corticosteroid patterns shown in the section on results.

These scanning patterns were a valuable step in the preliminary characterization of the corticosteroids for they provided a picture of the sequence of the following types of compounds:

1. UV absorbing, BT non-reducing - steroids with the Δ^4-3-ketone grouping intact and with no primary α-ketol group.
2. UV absorbing, BT reducing - steroids with both the Δ^4-3-ketone and primary α-ketol groups, e.g. F, E, B and S.

3. UV negative, BT reducing - steroids with reduction at ring A and with a primary α-ketol group, e.g. THF, THE and THS.

The areas under the curves also gave a rough estimation of the amounts of steroids in the different regions. However, there is not a strict proportion between area and amount of steroid, especially when the concentration of BT reducing material was very high, e.g. some zones of the compounds THF and THE.

D. Characterization of Corticosteroids.

The final step in this investigation was an attempt to carry out further characterization studies on the various metabolites making up the urinary corticosteroid patterns.

However, the scanning patterns were first carefully studied, in order to determine which compounds should be further studied, and then the appropriate zones of corticosteroid material were quantitatively eluted from the chromatograms with methanol. Aliquots of the various eluates were then submitted to one or more of the following procedures for their further characterization or, in some cases, fractionation.

1. Analytical Runs

In some cases the residues of these eluates were merely rechromatographed alongside the appropriate standards, each on separate narrow strips of paper, and in the appropriate solvent system. In these so-called analytical runs, the metabolites ran at the same rate as their respective standards (whereas the latter tended to move faster when greater amounts of urine extracts were chromatographed).

Thus, in such a way, an attempt was made to effect a clearer resolution of the corticosteroid mixtures at the regions of X_8, E-THS and B-S, by carrying out analytical runs on eluates of the first two zones in benzene-formamide and by rechromatographing the B-S regions in T-PG, undiluted.

2. Mixed Chromatograms

The Zaffaroni method (18) was used unmodified. If the urinary steroid tested with an appropriate standard was shown to have exactly the same running rate, it was presumed to be identical to the standard.

The technique was used to 'identify' some of the regularly occurring alpha-ketolic metabolites making up the sequence of compounds in the patterns of most patients. However, it was not seen necessary to re-establish this entire sequence of compounds in every set of patterns studied. No mixed chromatograms were done on patterns of subjects H.B., L.C. and of case W.R.

3. Sulphuric Acid Chromogen Spectra

In some cases, eluates of certain separated metabolites, after their further purification by the technique of the mixed chromatogram or acetylation, were submitted to treatment with concentrated H_2SO_4, according to the Zaffaroni method (115).

The spectra were taken on 0.5 ml. samples in microcuvettes with a Beckman spectrophotometer over the range from 220 to 620 mμ, against a blank of pure sulphuric acid. The optical densities were then plotted against the wavelength, and curves of maximum and minimum absorption were obtained.

The values of the absorbing maxima were then compared with those recorded in the literature, bearing in mind that the presence of impurities in the samples might cause a slight shift of the entire spectrum towards shorter wavelengths.

If the metabolite tested had the same chromatographic behaviour and absorbing maxima in H_2SO_4 as another authentic compound, then it was regarded as further proof that the two compounds were identical.

In this investigation the technique was used only in a few cases as a step for further characterization of metabolites already 'identified' by mixed chromatograms to be compounds THF, F and E. However, its most important use was in the identification of some of the components of the so-called X_8 complex of this study.

4. The Preparation and Chromatography of Acetates

These were prepared according to Zaffaroni (18). Three drops of acetic anhydride and 5 drops of pyridine were added to a small test-tube containing about 50 to 150 gamma of the dried steroid residue. Acetylation was permitted to occur overnight. The excess reagent was evaporated off under nitrogen below 50° C.

The acetates were chromatographed in the B-F system, either diluted or undiluted, until the solvent front reached the bottom. Appropriate free and acetylated standards were run at the same time. The positions of the various compounds were then detected by UV and BT spot testing.

This technique was used for the further study of a few metabolites separated on the patterns of the normal subjects and cases G.A., D.M. and F.T.

5. The Porter-Silber Reaction

The Porter-Silber (PS) reaction was carried out on aliquots of the eluates of various zones from the chromatograms.

Reagents

(i) H_2SO_4 solution - 310 cc. of concentrated H_2SO_4 were diluted to 500 cc. with distilled water.

(ii) Phenylhydrazine HCl solution - 65 mgm.% in the above acid solution, made freshly before use.

(iii) Methanol - distilled prior to use.

Technique

The macro method (81) was used essentially unmodified in order to determine the levels of THF combined with THE, F and E in the normal subjects and in case A.L.

A micro modification of this reaction was also used and is described briefly.

1. The corticosteroid material to be measured was divided equally (0 to 20 gamma of steroid in 0.2 cc. of methanol) among two sets of test tubes - (a) for color development, and (b) for urine blanks.
2. To tubes (a) 0.3 cc. of phenylhydrazine solution were added.
3. To tubes (b) 0.3 cc. of dilute acid solution were added.
4. Separate reagent and acid blanks were set up appropriately.
5. Reaction was permitted to occur at 60° C. for 60 minutes.

The optical densities of the colors developed were read at 405 mμ in a Beckman spectrophotometer.

All measurements were made against an acid blank and the optical densities were converted into gamma quantities by means of a factor obtained from a standard curve previously established.

This method was used for cases G.A., S.K. and F.T.

The Porter-Silber reaction in this investigation was used essentially as a characterization technique,in order to establish whether or not the steroid tested had a 17-hydroxyl group in addition to the primary alpha-ketolic structure. Therefore, all the results are referred to either as PS negative or PS positive. In addition, for cases A.L., G.A. and the normal subjects, the results have been calculated as mgm. of THF plus THE, F and E per 24 hours of urine excretion.

The quantitative determinations on the crude urine extracts were made in the routine endocrine laboratories for clinical purposes.

The 17-ketosteroids, 17-hydroxycorticosteroids, formaldehydogenic and 17-ketogenic steroids were measured according to the method of Callow and Callow (27), Porter and Silber (81), Daughaday (29), and Norymberski (2), respectively.

III. Clinical Material

A. Normal Subjects

These included a 38 year old male, H.B., and a 34 year old female, L.C. Single urine specimens were obtained from both subjects while they were receiving no therapy.

B. Adrenal Cortical Hypofunction Secondary to Anterior Pituitary Failure (Hypopituitarism)

1. Case D.M. was a 52 year old female with minimal to moderate hypopituitarism secondary to a suspected pituitary tumor which had been treated with deep x-ray therapy. The clinical findings included obesity, reduced body hair, spare axillary and pubic hair, hypertension - 230/120, a flat glucose tolerance curve, a B.M.R. within normal limits, and normal serum electrolytes. In addition, she developed rheumatoid arthritis 1 year before the present study.

The patient received cortisone, thyroid and oestrogen as replacement therapy.

The urine samples chromatographed, (a) and (b), were collected as follows:

(a) during the untreated disease state - total Porter-Silber chromogens (PS) and 17-ketosteroids (17-KS) were 1.4 and 0.1 mgm. per day, respectively.

(b) during the administration of 100 units of Armour ACTH, daily for 3 days - total PS and 17-KS were 2.02 and 1.4 mgm. per day, respectively.

2. Case A.L. was a 52 year old male with severe panhypopituitarism of 11 years duration. This followed upon partial surgical removal of a chromophobe adenoma. The clinical findings included marked weakness and fatigue, absent sexual and body hair, pale, thin skin, hypotension, a flat glucose tolerance curve, a low B.M.R. and normal serum electrolytes. The urinary PS and 17-KS values were approximately 0.3 and 0.9 mgm. per day, respectively.

This patient had been maintained on a variety of hormonal agents, including thyroid, testosterone, cortisone and ACTH.

The urine samples chromatographed, (a), (b) and (c), were collected during,

(a) the administration of 75 mgm. of cortisone per day - total PS and 17-KS values were 6.3 and 4.1 mgm. per day, respectively.

(b) the administration of 80 units of Nordic corticotropin (Acton) simultaneously with 75 mgm. of cortisone, per day - total PS and 17-KS values were 21.0 and 24.0 mgm. per day, respectively.

(c) the administration of Nordic long-acting ACTH (Duracton), 10 units per day - the values for total PS were 2.14 mgm. and 17-KS were 2.5 mgm., per day.

3. Case W.R. was a 61 year old male with moderate hypopituitarism of 10 years duration due to a large chromophobe

adenoma. The classical findings included weakness and fatigue, absence of body, axillary and pubic hair, striking pallor, low blood pressure, low B.M.R. and normal serum electrolytes. The urinary Porter-Silber chromogens and 17-KS were 0.17 and 1.6 mgm. per day, respectively.

The patient had received thyroid extract for many years, and was given 37.5 mgm. of cortisone acetate daily for 3 months, during which time the only urine sample chromatographed was collected - total PS and 17-KS were 6.12 and 5.6 mgm. per day, respectively.

C. Patients with Adrenocortical Hyperfunction

1. Case G.A. was a 27 year old female with classical Cushing's syndrome of 2 to 3 years duration. The clinical findings included moderate virilization, hypertension, diabetes, a normal serum sodium and chloride,but a low serum potassium and moderate osteoporosis.

Total adrenalectomy was done in 2 stages at 10 days interval and revealed bilateral adrenal cortical hyperplasia. Prior to removal of the first adrenal, 800 mgm. of cortisone were administered over a 3 day period. In the interval between the second adrenalectomy, the cortisone dosage was reduced,and three days before operation 200 mgm. of cortisone were given per day,intramuscularly.

Following adrenalectomy the symptoms of diabetes and hypertension disappeared and the patient was well, maintained

on 37.5 mgm. of cortisone daily.

Each of the three extracts chromatographed was obtained from pooled 3 day urines, collected as following:

(a) during the untreated disease state - free formaldehydogenic steroids (FSS) and 17-KS were 2.64 and 35 mgm. per day, respectively.

(b) during the administration of 100 units of Armour corticotropin daily for 3 days - free FSS ranged from 14.30 to 18.70 mgm. per day and 17-KS increased to 120 mgm. per day.

(c) on the 7th, 8th, and 9th days after the first adrenalectomy, when the cortisone dosage was 200 mgm. per day - free and conjugated FSS were 2.78 and 47.8 mgm. per 24 hours, respectively.

2. Case S.K. was a 60 year old female with classical Cushing's syndrome of 8 to 9 years duration. The clinical findings included minimal virilization, no hypertension, a diabetic glucose tolerance curve, normal serum electrolytes, and marked generalized osteoporosis.

A one stage total adrenalectomy was performed which revealed bilateral adrenal cortical hyperplasia. Preoperatively, 700 mgm. of cortisone acetate were given over a 3 day period, and the cortisone dosage was gradually reduced to 50 mgm. per day.

The urine samples chromatographed were collected as follows:

(a) during the untreated disease state - total PS and 17-KS were 9.5 and 25.5 mgm. per day, respectively.

(b) during the administration of 80 units of Nordic corticotropin (Acton X), daily for 2 days - total PS and 17-KS were from 16.9 to 18.7 and 43.5 mgm. per day, respectively.

(c) after total adrenalectomy, when the patient was receiving 50 mgm. of cortisone per day - total PS were 4.3 mgm., and 17-KS were 14.6 mgm., per day.

3. Case F.T. was a 14 year old male with congenital adrenal hyperplasia complicated by episodes of hypertension, during which time there was a severe headache. The patient also had suffered from bouts of intense abdominal pain since the age of 5 years. The clinical history revealed that at 9 years of age the child had hirsutism, advanced muscular and bone development, and other signs of premature masculinity. Present laboratory findings showed the serum sodium, potassium and chloride to be 129, 4.35 and 103 mEq. per litre, respectively. The blood pressure in the intervals between attacks was 130/90.

While under no treatment, the urinary levels of the 17-KS ranged from 21.94 to 48.1 mgm. per day, while the

17-KGS (ketogenic steroids) ranged from 55 to 93 mgm. per day. Normal values are 4.0 mgm per day. The plasma Porter-Silber chromogens were initially 30 gamma per 100 ml.

During the intravenous administration of ACTH, the 17-KS values were unchanged but the 17-KGS decreased to 6 mgm. per day. Just prior to the test, the plasma PS values were 38.12 gamma, but during ACTH administration the values decreased to 31.02 gamma per litre.

During the intravenous infusion of 75 mgm. of cortisone over a period of 18 hours, the 17-KGS increased to 79 mgm. per day, while the 17-KS decreased from 40.3 to 13.06 mgm. per day. This was followed by the oral administration of 50 mgm. of cortisone per day, during which the 17-KS and 17-KGS decreased to 4.09 and 11.18 mgm. per day, respectively, while the plasma corticosteroids were 24.3 gamma per 100 ml.

The patient is now maintained in an improved condition on 50 mgm. of cortisone per day.

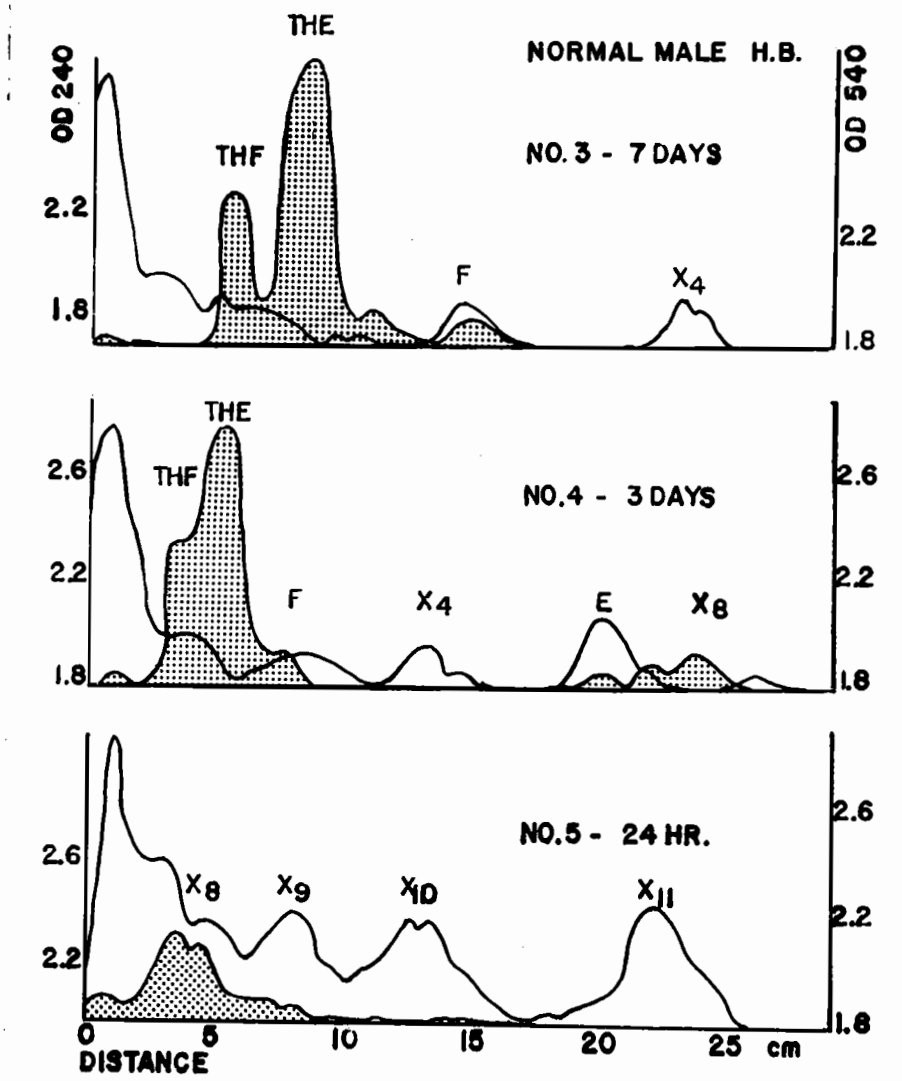
Urine samples were collected for chromatography (a) during the untreated disease state and (b) during cortisone therapy.

IV. Results

The scanning patterns and the results of characterization studies in tabled form are shown together. Prior to the actual description of the results, a brief explanation of the types of corticosteroids applied on each chromatogram is given with a statement of the concentration used.

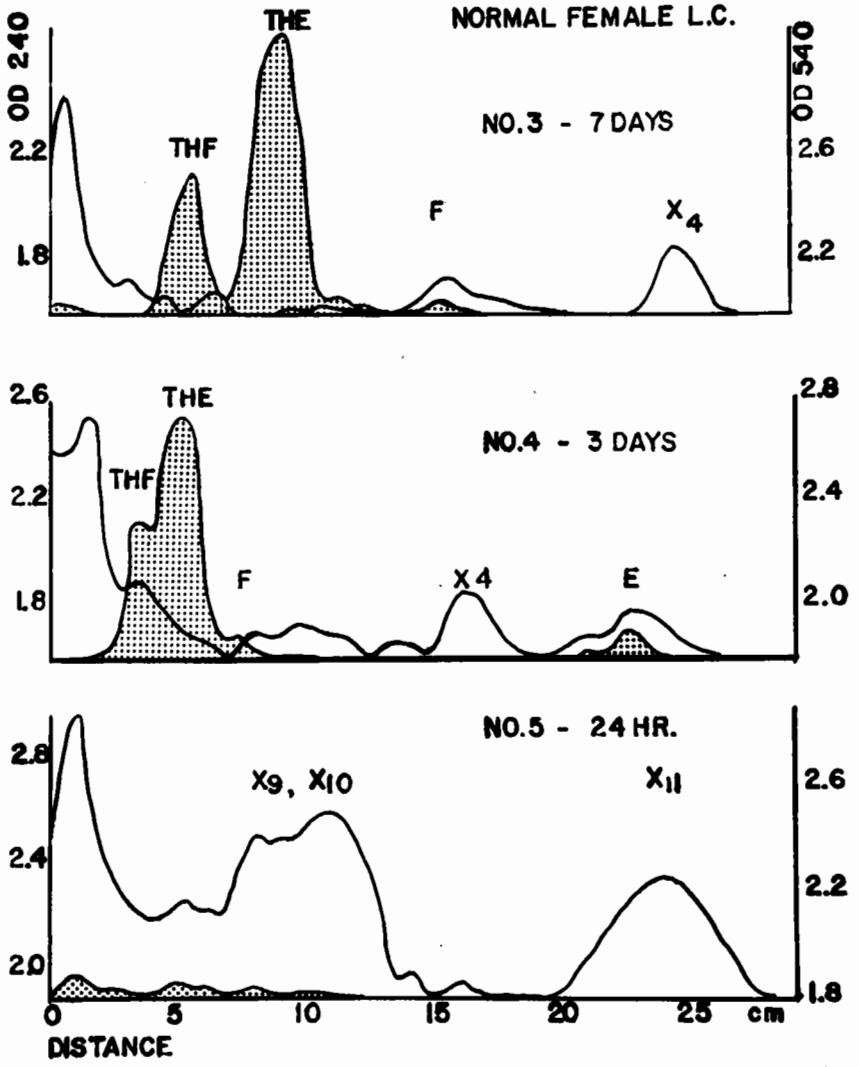
The regularly occurring urinary corticosteroids, such as tetrahydro-F, tetrahydro-E, compounds F and E, are considered as 'identified' by their chromatographic behavior, UV absorption, BT reduction and H_2SO_4 chromogen spectra. The latter is given for tetrahydro-F and tetrahydro-E in Table II for compounds E and F in Table XI. The results of studies carried out on any other compounds such as X_8 are described in the text. It is important to say at this point that the general designation X_8 has been given to the series of alpha-ketolic corticosteroids which run between compounds E and S in the toluene-propylene glycol system, but which are very efficiently separated from cortisone in the benzene-formamide system. Thus, the designation X_8 is meant to indicate that at least one or more of the reduced metabolites of the $C_{21}O_4$ corticosteroids, such as compounds S, B and A, is present. These metabolites, listed in an order of decreasing polarity, are the compounds tetrahydro-S, tetrahydro-B, allotetrahydro-B and tetrahydro-A. It was rather difficult

FIGURE 1.



Normal subject H.B. scanning patterns of the urinary corticosteroids ranging from polarity of compounds THF to below F on 7 day chromatogram; from polarity of compounds THF to X_8 on the 3 day chromatogram and of all the $C_{21}O_4$ corticosteroids on the 24 hr. chromatogram.
Concentration applied was 0.8 hr urine aliquot per cm.

FIGURE 2.



Normal subject L.C. scanning patterns of the urinary corticosteroids ranging from polarity of compounds THF to below F on 7 day chromatogram; from polarity of compounds THF to X_8 on the 3 day chromatogram and of all the $C_{21}O_4$ corticosteroids on the 24 hr. chromatogram.
Concentration applied was 0.8 hr. urine aliquot per cm.

Characterization studies carried out
on corticosteroids isolated from urine

TABLE I. Subject H.B

Compound	Source chrom. no.	eluted dist., cm	UV	BT	PS mgm./24 hours	Mobility of the compound acetate
THE	3	4-6.5	-	+	2.2	
THE	"	6.5-11	-	+		
F	"	13-17	+	+	0.1	
X_4	"	21-26	+	-		runs between acetates of compounds F and E
E,X_8	4	19-27	+,-	+,+	0.16	
X_8	5	0-10	+	+		
X_9	"		+	-		
X_{10}	"		+	-		
X_{11}	"	19-25	+	-		*

*X_{11} does not acetylate - on rechromatography, it was shown to consist of 2 UV+, BT- compounds

Characterization studies carried out
on corticosteroids isolated from urine

TABLE II. Subject L.C.

Compound	Source chrom. no.	eluted dist/cm	UV	BT	PS, mgm /24 hours	Maxima of H_2SO_4 chromogen Spectra unknown/ standard
THF	3	4-6.5	-	+	1.6	THF= 320,419,500 / 330,415,510
THE	"	7-11	-	+		THE = 268,320,410 / 270,335,410
F	"	13-18	+	+	0.5	
X_4	"	23-26	+	-		
E	4	20-25	+	+	0.8	
X_9, X_{10}	5		+	-		
X_{11}	"	19-27	+	-		

to distinguish these even after their separation upon re-chromatography. They do not absorb UV light but reduce BT, and care must be taken in the interpretation of their respective H_2SO_4 chromogen spectra as they are very similar. For absolute identification of the tetrahydro derivatives infrared spectrophotometry is essential. Therefore, although in a number of cases some of the components of Xg had been characterized, it was considered better not to designate them specifically tetrahydro-S, tetrahydro-B, etc., upon the chromatogram, but rather to refer to them as Xg.

A. Normal Subjects

The urinary corticosteroid pattern of two normal subjects receiving no therapy was studied, Figures 1 and 2. The more polar $C_{21}O_5$ corticosteroids, ranging in polarity from tetrahydro-F to below compound F, are well separated upon the 7 day chromatogram. The scanning pattern of the 3 day chromatogram shows these same corticosteroids, but with practically no separation close to the starting line, while cortisone and any faster-moving corticosteroids, Xg, ranging in polarity from that of tetrahydro-S to that of approximately tetrahydro-B, are separated over the remainder of the chromatogram. The scanning pattern of all the $C_{21}O_4$ corticosteroids, including Xg, is shown on the 24 hour chromatogram. The concentration of material applied on each of the chromatograms was 0.8 hours

urine aliquot per cm. width of paper, thus allowing easy comparison of the relative amounts of the various corticosteroids.

The patterns, Figures 1 and 2, were very similar and are described together. The results of characterization studies for H.B. are included in Table I and for L.C. in Table II.

In both subjects, by far the bulk of the alpha-ketolic steroid material excreted consisted of the regularly occurring metabolites tetrahydro-F and tetrahydro-E. What is striking in both subjects is that the levels of tetrahydro-E are considerably greater than those of tetrahydro-F, Figures 1 and 2. The output of these metabolites, combined, was estimated by Porter-Silber color reaction on the eluates to be 2.2 and 1.6 mgm. per 24 hours for the male and female subjects, respectively.

The biologically active corticosteroids, compounds E and F, were present in both normal patterns but in very small amounts and in each case the level of cortisone was greater than that of hydrocortisone. Compound F was estimated to be excreted at the rate of 0.05 and 0.1 mgm. per 24 hours for L.C. and H.B., respectively, while the material at the region of cortisone and cortisone plus X_8, for the female and male pattern was estimated to be 0.08 and 0.16 mgm. per 24 hours, respectively.

The compound X_4 was seen in approximately the same amounts in both patterns. It is a UV absorbing substance which on acetylation had the chromatographic behaviour of a monoacetate in benzene-formamide.

Thus the male and female patterns were essentially similar, both qualitatively and quantitatively, with respect to $C_{21}O_5$ urinary corticosteroid excretion. However, there was a difference in the amount of $C_{21}O_4$ corticosteroids excreted in these two normal subjects. While the male pattern, Figure 1, showed very small but detectable amounts of the compound X_8 running below cortisone, there was no corresponding peak in the pattern of L.C., Figure 2. This difference in amount of $C_{21}O_4$ corticosteroid excretion was re-illustrated on the 24 hour chromatogram, since significant amounts of X_8 and traces of less polar metabolites were observed only in the male pattern.

The series of UV absorbing, BT non-reducing metabolites, characteristically found on the 24 hour chromatograms, X_9, X_{10} and X_{11} were present in approximately the same amounts in both subjects. The metabolite X_{11} did not form an acetate. Upon rechromatography in benzene-formamide, it was shown to consist of 2 compounds. The more polar component when treated with concentrated H_2SO_4 absorbed UV light maximally from 220 to 310 mμ, but between 310 and 620 mμ there were no distinct absorbing maxima.

Thus in both normal subjects the major urinary metabolites were tetrahydro-F and tetrahydro-E while relatively very small amounts of the biologically active corticosteroids, compounds F and E, were excreted. There were significant amounts of the less polar, $C_{21}O_4$, corticosteroids only in the male pattern studied.

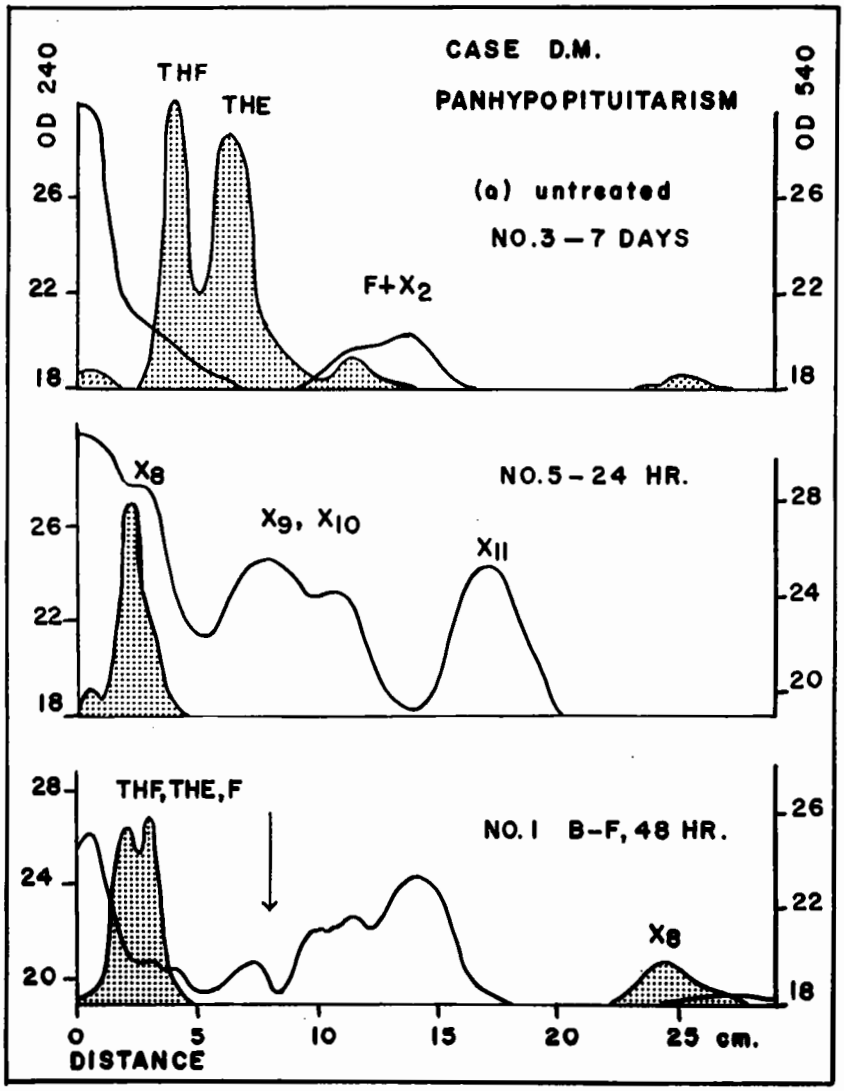
B. Patients with Adrenal Cortical Hypofunction Secondary to Anterior Pituitary Failure (Hypopituitarism)

Case D.M.

The corticosteroid patterns on this patient with hypopituitarism are shown in Figures 3 and 4. Figure 3 and Table III represent the results on the urine when the patient received no therapy (a). Figure and Table IV show the results on the urine while the patient received ACTH, (b).

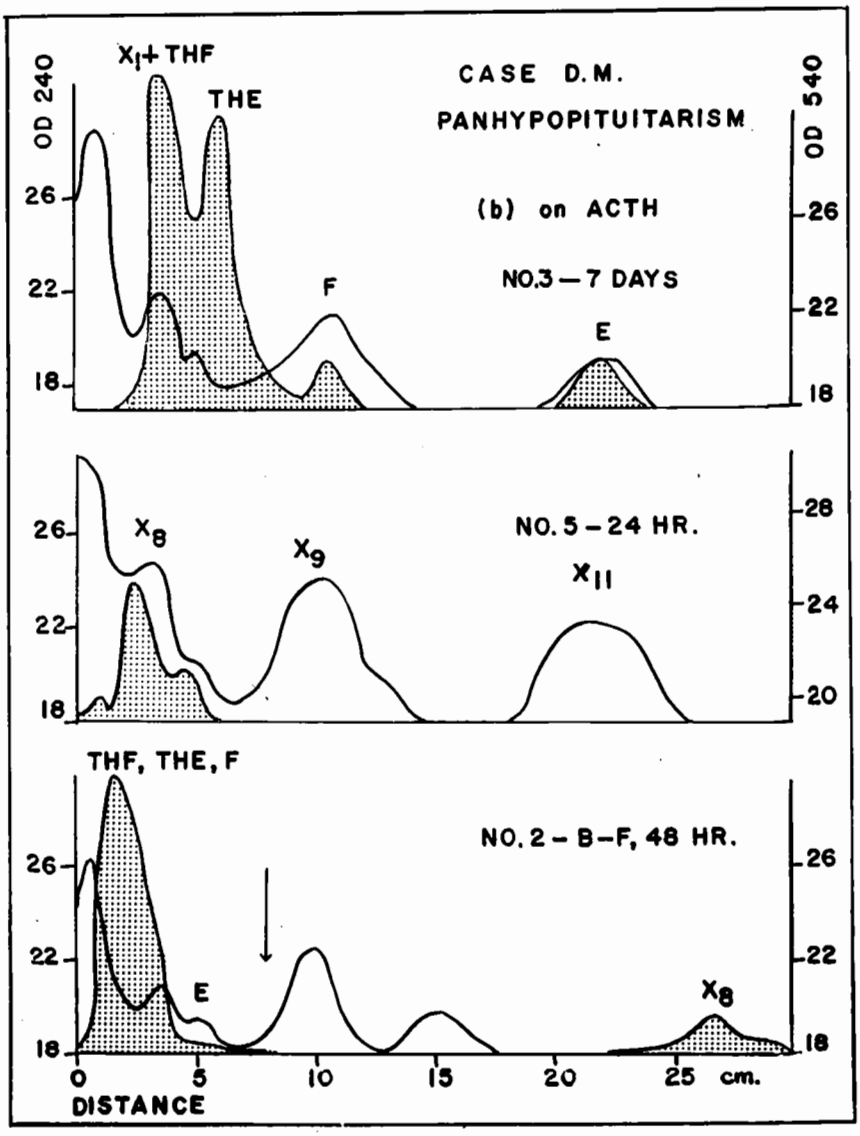
Since the scanning patterns of the $C_{21}O_5$ corticosteroids, ranging in polarity from tetrahydro-F to cortisone are seen on the 7 day chromatogram, and those of any X_8 and less polar $C_{21}O_4$ corticosteroids are present on the 24 hour chromatogram, it was not necessary to show the scanning pattern of the 3 day chromatogram. However, the pattern of the initial benzene-formamide papergrams is shown. On comparing these patterns with those of the normal subjects one should recall that, although 0.8 hour urine aliquot per cm. width of strip was applied on the benzene-formamide papergrams, as for the normal subjects, the concentration used on the toluene-propylene glycol papergram was 1.9 hours per cm. - more than double that used for the normals, because

FIGURE 3.



Case D.M. (a) untreated hypopituitarism, scanning patterns of the urinary corticosteroids ranging from polarity of compounds THF to below E on the 7 day chromatogram; of all the $C_{21}O_4$ corticosteroids on the 24 hr. chromatogram; of compounds THF to X8 on the benzene formamide chromatogram. Concentration applied was 1.9 hr. urine aliquot per cm. on the 7 day and 24 hr. chromatogram and on the benzene formamide 0.8 hr.

FIGURE 4.



Case D.M. (b) hypopituitarism on ACTH administration, scanning patterns of the urinary corticosteroids ranging from polarity of compounds THF to below E on the 7 day chromatogram; of all the $C_{21}O_4$ corticosteroids on the 24 hr. chromatogram; of compounds THF to X_8 on the benzene formamide chromatogram.
Concentration applied was 1.9 hr. urine aliquot per cm. on the 7 day and 24 hr. chromatogram and on the benzene formamide 0.8 hr.

Characterization studies carried out
on corticosteroids isolated from urine

TABLE III. Untreated hypopituitarism
Case D.M., pattern (a)

Compound	Source chrom. no.	eluted dist/cm.	UV	BT	Mobility of compound free	acetate
THF	3	3-5	-	+	runs with THF	
THE	"	5-7	-	+	runs with THE	
F + X_2	"	10-16	+	+		
X_8	5	0-5	+	+		1) UV+BT?, slower than Sac 2) UV+BT-, runs with Sac 3) UV-BT+ runs with solvent front
X_8	1		-	+		
X_9, X_{10}	5		+	-		
X_{11}	"		+	-		

Characterization studies carried out on corticosteroids isolated from urine.

TABLE IV

Hypopituitarism on ACTH administration, Case D.M. pattern (b)

Compound	Source chrom. no.	eluted dist/cm.	UV	BT	Mobility of compound free	acetate	Maxima of H_2SO_4 chromogen spectra unknown /standard
X_1+THF	3	0-5	+,-	+			290,330,415,510 [*1] / THF= 330,415,510
THF	"		-	+			
F	"	9.5-12	+	+	runs with F		
E	"	19-25	+	+	runs with E		
X_8	5	0-6	+	+		1) UV+BT- faster than E ac 2) UV+BT- faster than E ac 3) UV+BT- slower than S ac 4) UV-BT+moved with solvent front	
X_8	2		-	+			
X_9	5		+	-			
X_{11}	"	19-25	+	-			

[*1] - The maxima obtained at 290 must be due to the admixture of the UV light absorbing compound X_1, which compounds generally show great maxima between 280 and 290 mu.

only small amounts of corticosteroids were expected in the urine of this patient.

The greatest amount of corticosteroid material in both patterns (a) and (b) was present as compounds tetrahydro-F and tetrahydro-E. Only slight differences can be detected in the pattern of tetrahydro-F and tetrahydro-E after ACTH - the levels were somewhat greater (this is better illustrated on the benzene formamide papergrams). The control pattern showed somewhat larger amounts of tetrahydro-E than of tetrahydro-F. Following administration of ACTH there was only a slight increase in the amount of tetrahydro-F as compared to that of tetrahydro-E.

The use of the greater concentration per cm. permitted the detection of hydrocortisone which was present in only very small amounts in both patterns in contrast to the amounts of the reduced metabolites. Prior to ACTH, only negligible amounts of alpha-ketolic material were present in the region of cortisone in the control pattern (too little to give a good mixed chromatogram), while on ACTH administration the cortisone peak was greater than that of hydrocortisone.

The metabolite X_4 was not present in detectable amounts in either of the patterns.

The qualitative and quantitative pattern of the less polar steroids was essentially unchanged on ACTH administration.

This is well shown by a study of both the toluene-propylene glycol and benzene-formamide papergrams. Upon acetylation and rechromatography, the bulk of the X_8 complex was shown to have no UV light absorption and to possess at least 2 acetylable hydroxyl groups. There were traces of less polar $C_{21}O_4$ corticosteroids, one of which might have been compound S according to the rate of flow of its acetate. Certainly ACTH administration did not result in the appearance of detectable amounts of B-like compounds on the chromatogram, in contrast to the increased amount of cortisone observed.

It is also obvious that ACTH had no effect in altering the pattern of the UV light absorbing metabolites X_9, X_{10} and X_{11} which were also demonstrated in the normal patterns, Figures 1 and 2.

The benzene-formamide papergram from this patient is of interest as it illustrates the difference in chromatographic behaviour of the $C_{21}O_5$ and the $C_{21}O_4$ corticosteroids in this system after 48 hours of development. The former, ranging from tetrahydro-F to cortisone, were retained close to the starting line, while the latter developed much more quickly. In this period some of the less polar constituents of X_8 had

overflowed off the papergram, but the bulk of Xg remained toward the end of the paper. Hence, a truer picture of Xg was seen on the benzene-formamide papergram, showing it to have consisted of BT reducing steroids with no UV light absorption. The major part of the UV absorption at the region of Xg in the 24 hour chromatogram may be regarded as due to 'contaminating' non-alpha-ketolic material,which separated from Xg in benzene-formamide (having come off in the 5 to 48 hour overflow), but when reapplied with Xg in toluene-propylene glycol it had the same chromatographic behaviour as the latter.

In summary, pattern (a) of the untreated disease state was qualitatively very similar to the normal pattern, but all compounds occurred in much smaller quantities. Pattern (b) illustrated that this patient responded poorly to ACTH administration, as only slightly greater amounts of tetrahydro-F and cortisone were excreted in the urine, whereas tetrahydro-E, compound F, and the compounds less polar than cortisone appeared to remain unaffected by the ACTH.

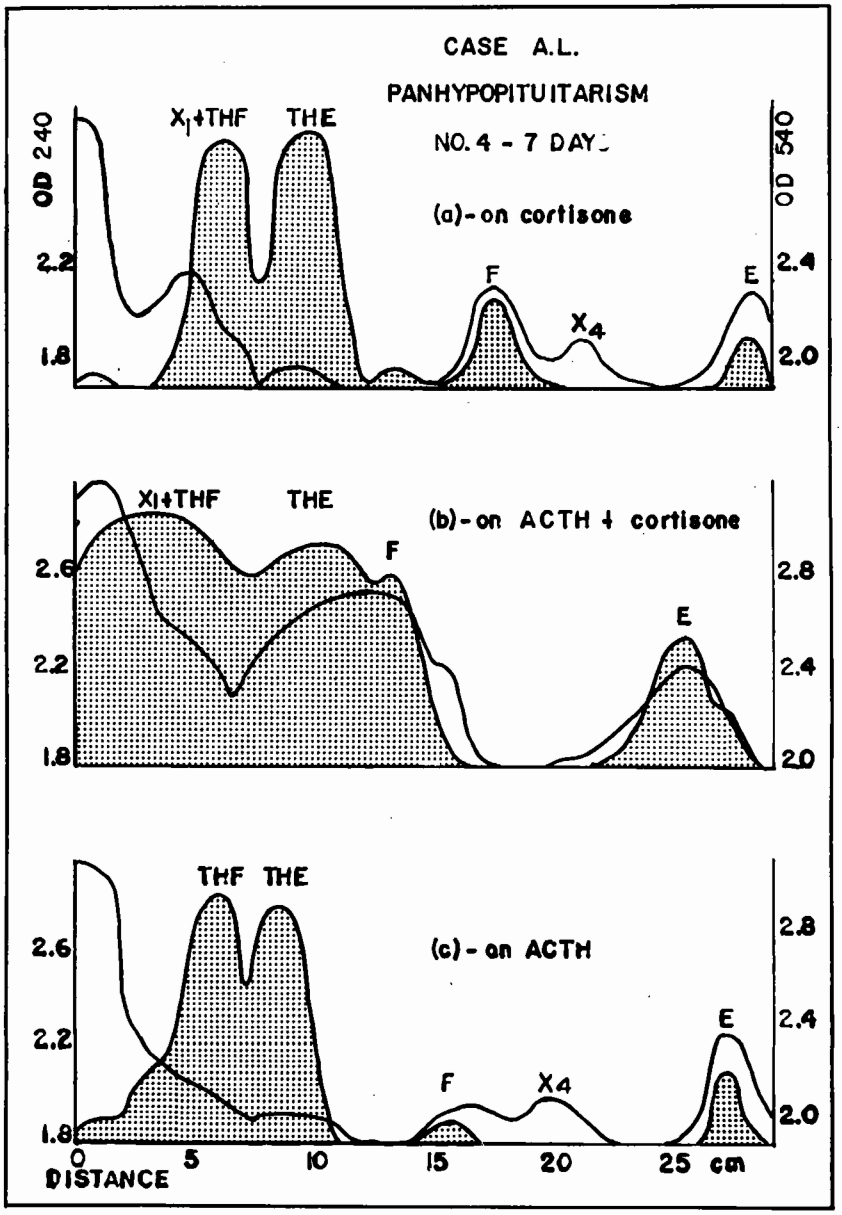
Case A.L.

Case A.L., a patient with severe hypopituitarism, was studied only while he was on therapy. No 'control' chromatogram was obtained as it was considered unwise to discontinue

treatment at the time this study was made. Certain laboratory findings should be mentioned in order to make the following results more meaningful. The endogenous urinary corticosteroid excretion, when later measured by the Porter-Silber technique, was found to be very low - averaging 0.3 mgm. per 24 hours. Hence, the pattern of extract (a), with respect to the excretion of 17-hydroxycorticosteroids, might be considered to be essentially that of the metabolites of the administered cortisone - 75 mgm. orally per day. Also, since for extract (b) this same cortisone dosage was given simultaneously with a Nordic ACTH preparation, any significant differences in patterns (a) and (b) can be attributed to the effects of the ACTH. Pattern (c), on the other hand, illustrates the degree of stimulation of residual adrenal cortical activity, as evaluated by urinary corticosteroid excretion, to another ACTH preparation (Duracton) when given alone.

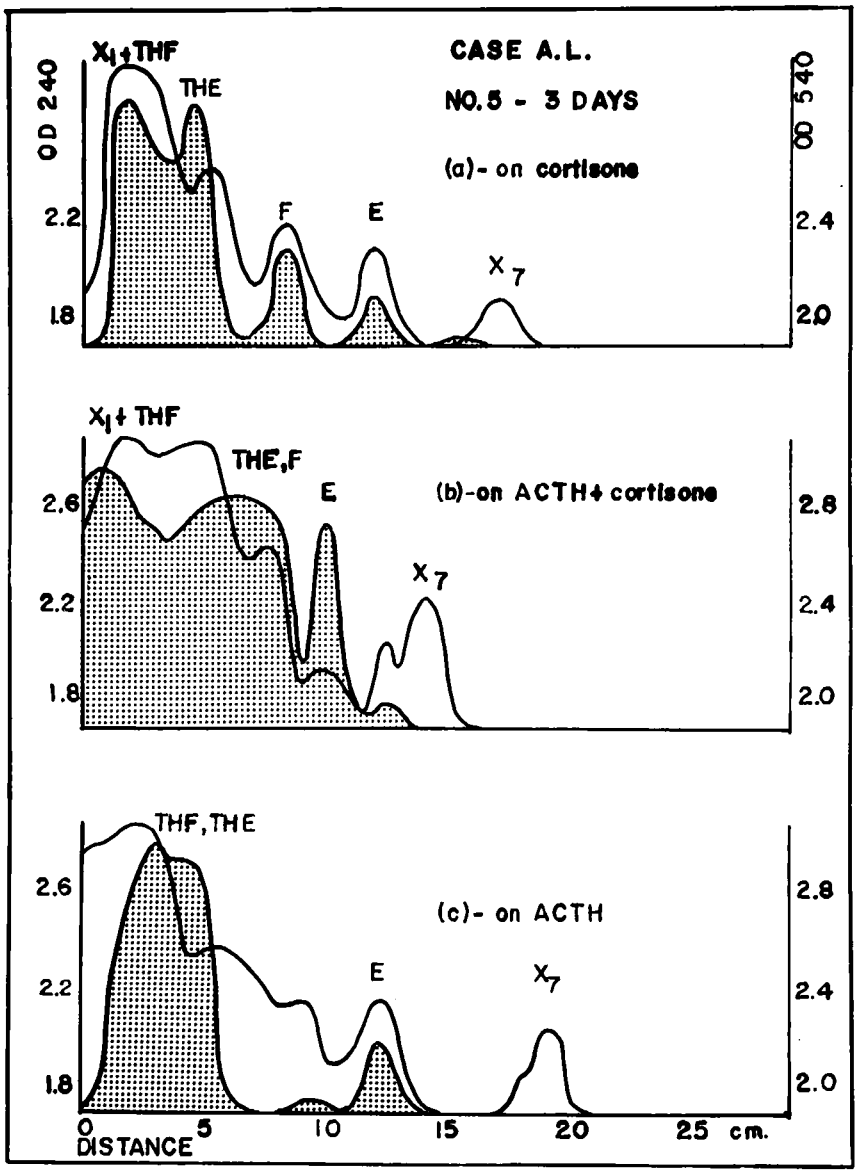
In this case, the pattern of the corticosteroids ranging in polarity from tetrahydro-F to compound F was duplicated on the 7 and 3 day chromatograms, Figures 5 and 6. The characterization studies were in most part carried out on eluates of the 7 day paper, but the 3 day chromatogram is shown in order to illustrate the importance of development time for the resolution of the $C_{21}O_5$ corticosteroids in the toluene-propylene glycol system. The scanning pattern of all the

FIGURE 5.



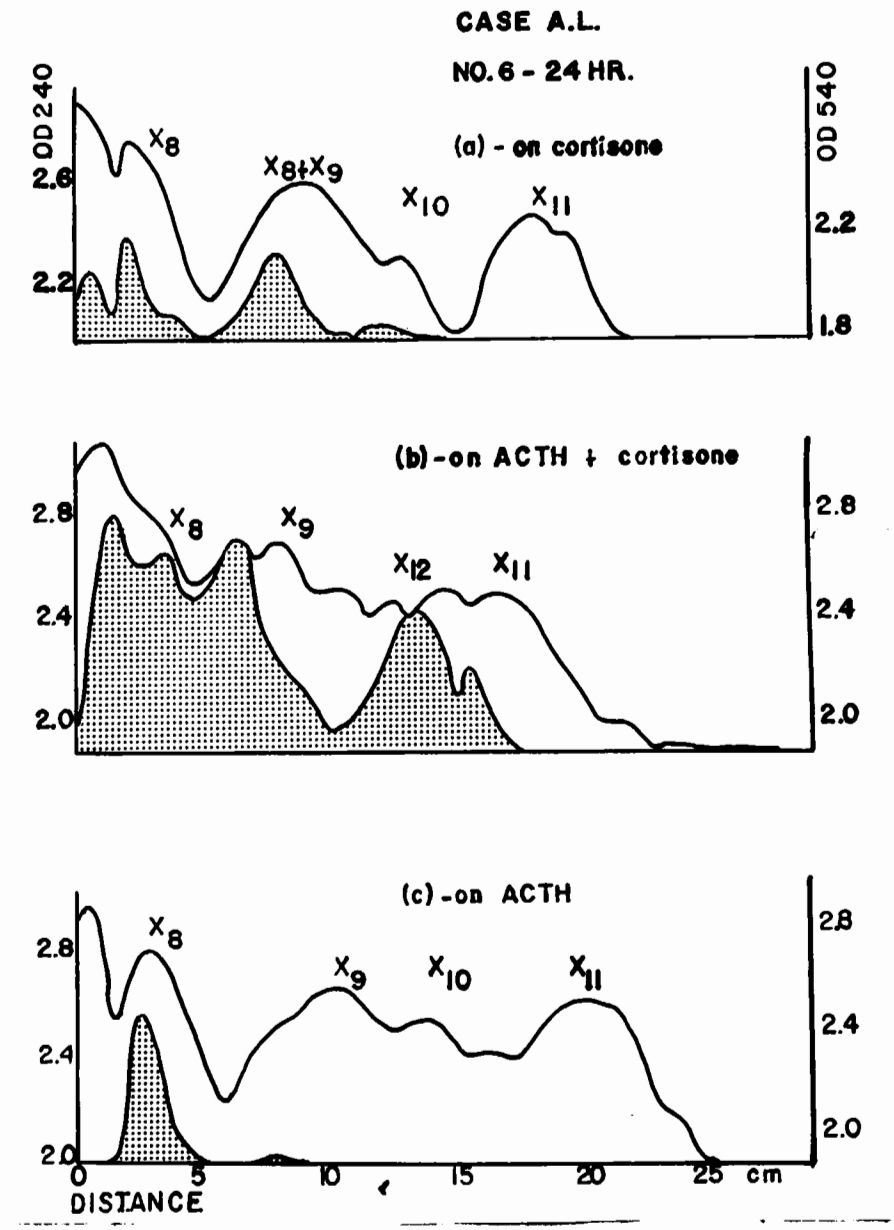
The scanning patterns of the urinary corticosteroids ranging in polarity from compounds THF to E on the 7 day chromatogram for case A.L.,a patient with hypopituitarism;(a) on cortisone (b) on both ACTH and cortisone and (c) on a different ACTH preparation, given alone.
Concentration applied was 1.4 hour urine aliquot per cm.

FIGURE 6.



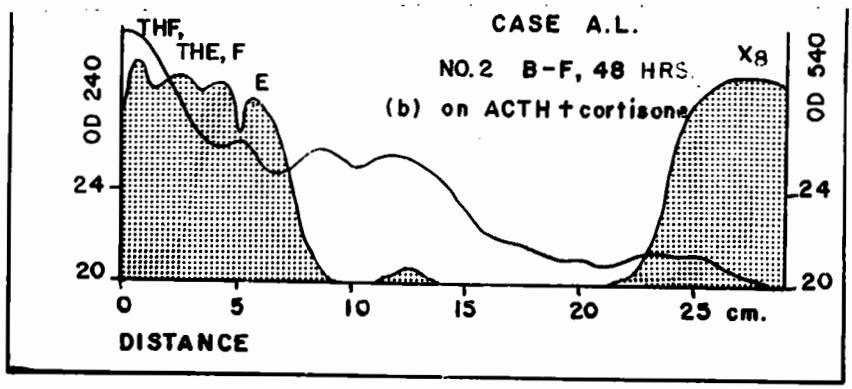
The scanning patterns of the urinary corticosteroids ranging in polarity from compounds THF to E, on the 3 day chromatogram, for case A.L., a patient with hypopituitarism; (a) on cortisone, (b) on both ACTH and cortisone and (c) on a different ACTH preparation, given alone.
Concentration applied was 1.4 hour urine aliquot per cm.

FIGURE 7.



The scanning patterns of the $C_{21}O_4$ urinary corticosteroids ranging in polarity from below compound E to below compound B, on the 24 hr chromatogram, for case A.L., a patient with hypopituitarism; (a) on cortisone, (b) on both ACTH and cortisone and (c) on a different ACTH preparation, given alone. Concentration applied was 1.4 hour urine aliquot per cm.

FIGURE 8.



The scanning pattern of the urinary corticosteroids ranging in polarity from compound THF to below compound THS, on the initial benzene-formamide chromatogram, for case A.L., a patient with hypopituitarism on both ACTH and cortisone administration. Concentration applied was 0.8 hour urine aliquot per cm.

Characterization studies carried out on corticosteroids isolated from urine.

TABLE V

Hypopituitarism, on cortisone administration, Case A.L., pattern (a)

Compound	Source chrom. no.	eluted dist./cm	UV	BT	PS	mgm/24 hours	Mobility of compounds free
X_1 +THF	4	3-7	+,-	+		4.6	runs with THF
THE	"	7-12	-	+			runs with THE
F	4	15-20	+	+	+		runs with F
X_4	"		+	-			
E	5	10-14	+	+	+		
X_7	"		+	-			
X_8	6	1-4	+	+	+		
X_8 +X_9	"	6-10	+	+	-		
X_{10}	"		+	-			
X_{11}	"		+	-			

Characterization studies carried out on corticosteroids isolated from urine.

TABLE VI

Hypopituitarism, on cortisone and ACTH administration, Case A.L., pattern (b)

Compound	Source chrom. no.	eluted dist.cm.	UV	BT	PS mgm.24 hr.	Mobility of compounds free	acetate	Maxima of H_2SO_4 chromogen spectra unknown/standard
X_1 + THF	4	0-7	+,-	+	16.32	runs with THF		
THE	"	7-12	-	+		runs with THE		
F	"	12-16	+	+	+	runs with F		
E	"	21-29	+	+	0.44	runs with E		
X_7	5		+	-				
*1 X_8 + X_9	6	0-10	+	+		1) UV-BT+, runs with THS 2) UV-BT+, runs faster than THS 3) UV+BT- runs faster than THS 4) UV+BT- runs faster than THS		310, 320, 410 THS= 315, 410
*2 X_{12}	"	10-19	+	+				
X_{11}	"		+	-				
X_8	2	21-30	-	+				

*1 includes a compound which has been characterized to be tetrahydro-S

*2 X_{12} - a UV-BT+component has maxima of H_2SO_4 chromogen spectrum at 310, 370, 400

Characterization studies carried out on corticosteroids isolated from urine

TABLE VII

Hypopituitarism, on ACTH administration, Case A.L., pattern (c)

Compound	Source chrom. no.	eluted dist. cm.	UV	BT	PS	mgm/24 hour	Mobility of compounds free
THF	4	0-7	-	+		1.9	runs with THF
THE	"	7-11	-	+			runs with THE
F	"	14-17	+	+	+		runs with F
X_4	"		+	-			
E	5	11-14	+	+		0.07	runs with E
X_7	"		+	-			
X_8	6		+	+			
X_9	"		+	-			
X_{10}	"		+	-			
X_{11}	"		+	-			

$C_{21}O_4$ corticosteroids present in the extracts was obtained on the 24 hour chromatogram, Figure 7. It was also of interest to show the pattern of the initial B-F papergram, Figure 8. The concentration of corticosteroid material applied on the toluene-propylene glycol papergrams was not quite double that used for the normals, 1.4 hour urine aliquot per cm. width of strip, while the usual 0.8 hour per cm. was applied on the benzene-formamide papergram.

The results of the three urinary corticosteroid patterns studied are presented separately but comparisons are made throughout the description.

The scanning patterns for case A.L. on cortisone administration are shown in Figures 5 to 8 and the results of characterization studies are given in Table V.

The metabolites tetrahydro-F and tetrahydro-E were by far the major compounds in pattern (a). They were estimated by the Porter-Silber reaction to be excreted at the rate of 4.6 mgm. per 24 hours.

The amounts of hydrocortisone excreted were very significant and certainly greater than those of cortisone.

The metabolite X_4 was excreted in relatively minor amounts.

As mentioned previously, the entire pattern of the $C_{21}O_5$ corticosteroids was reillustrated on the 3 day chromatogram with a lesser extent of separation. In the pattern (a) there was a UV light absorbing, BT reducing metabolite, below cortisone designated as X_7, which was slower or had the same chromatographic behaviour in benzene-formamide as did cortisone but when rechromatographed in toluene-propylene glycol it was faster.

No extensive characterization studies were carried out on the metabolite designated simply as X_8 on the pattern. However, it was shown to be quantitatively Porter-Silber positive while the less polar component of the X_8 complex was not. It is very likely that small amounts of tetrahydro-B and tetrahydro-A may have been components of this alpha-ketolic steroid mixture, although they were not identified.

The characteristically occurring urinary metabolites X_9, X_{10} and X_{11} were all present in significant amounts on the pattern.

The scanning patterns of case A.L. on both cortisone and ACTH administration are shown in Figures 5 to 8 and the results of characterization studies are included in Table VI.

On the administration of both 75 mgm. of cortisone and ACTH the levels of tetrahydro-F and tetrahydro-E excreted in the urine were greatly increased. Porter-Silber estimation on these compounds combined was 16.32 mgm. per 24 hours as contrasted to the value of 4.6 mgm. obtained on the administration of cortisone alone. In fact, the concentration of alpha-ketolic material at the positions of tetrahydro-F and tetrahydro-E was so great that there was no longer any proportion between the size of the BT peak and the actual amount of BT reducing material.

The levels of hydrocortisone and cortisone excreted in the urine increased correspondingly. The former compound was present in amounts too great to permit separation from the bulk of tetrahydro-F and tetrahydro-E with the concentration applied. A study of patterns (a) and (b) in Figure 5 shows

the effect of this combination of hormone therapy very strikingly.

No significant amounts of X_4 were present on the pattern but very striking is the great increase of UV light absorbing material at the region of tetrahydro-F, due to the presence of compound X_1, when both ACTH and cortisone were given. An attempt to separate X_1 from tetrahydro-F by acetylation was not successful as the UV light absorbing material moved with the solvent front in benzene-formamide together with tetrahydro-F. However, the H_2SO_4 chromogen spectrum carried out on this unseparated tetrahydro-F,X_1 acetate mixture was similar to that of authentic tetrahydro-F, alone.

The metabolite X_7 can be seen in Figure 6 in amounts greater than in pattern (a), i.e., on the administration of cortisone alone.

This hormone therapy also brought about marked changes in the pattern of $C_{21}O_4$ corticosteroids as can be seen in Figure 7. It must be recalled that the method of chromatography used permitted the appearance of the entire X_8 complex on the 24 hour chromatogram. As a result, tetrahydro-S had been shown

to be a component of the mixture of the more polar corticosteroids in the region of X_8 plus X_9, on the basis of chromatographic behaviour in toluene-propylene glycol and benzene-formamide and H_2SO_4 chromogen spectrum - the absorbing maxima and shape of the curve were identical to those of the authentic compound. This X_8 complex also consisted of at least 1 other less polar BT reducing metabolite with no UV light absorption which was possibly tetrahydro-B. The alpha-ketolic material at the region of X_{12} was rechromatographed in benzene-formamide. The greater part of X_{12} did not absorb UV light and had absorbing maxima of 310, 370,400 in concentrated H_2SO_4. Initially it was believed to be the reduced metabolite of compound A but the H_2SO_4 chromogen spectrum obtained was different. Small amounts of any of the compounds B, S or A may have been present but did not show separately since there was too much reduced material in this combined free and enzyme hydrolysed fraction applied. The most significant aspect, however, was the very marked increase in the urinary excretion of the $C_{21}O_4$ corticosteroids when both ACTH and cortisone were administered so that their relative amount approached that of $C_{21}O_5$ corticosteroids excreted in the urine.

The scanning pattern of the initial benzene-formamide papergram for extract (b), Figure 8, shows this very well. In this system the $C_{21}O_5$ corticosteroids were found close to the starting line while only the more polar components of X_8 were retained on the paper. Thus, there could be seen a great amount of alpha-ketolic material in both the regions of the $C_{21}O_5$ and $C_{21}O_4$ corticosteroids.

The series of metabolites, X_9, X_{10} and X_{11}, did not seem to have increased in proportion to the other compounds described. Instead they were present in relatively the same amounts as in pattern (a).

The scanning patterns for case **A.L.** on administration of another ACTH preparation are shown in Figures 5 to 8 and the results of characterization studies are given in Table VII.

It could be seen that when cortisone was withdrawn and the patient carried on a different long-acting ACTH preparation the levels of the metabolites tetrahydro-F and tetrahydro-E excreted in the urine were significantly decreased

in comparison with the amounts present in extracts (a) and (b). A value of 1.9 mgm. per 24 hours for these compounds combined was estimated by the Porter-Silber technique. Nevertheless, it was apparent that tetrahydro-F and tetrahydro-E still formed the bulk of the alpha-ketolic steroid material excreted in the urine.

When ACTH was given alone the biologically active corticosteroid hydrocortisone was barely detectable on the chromatogram but the levels of cortisone were significantly greater. The latter compound was excreted at the rate of 0.07 mgm. per 24 hours. It could be seen that there was a tendency for the opposite to be true when cortisone alone was administered, pattern (a).

The metabolite X_4 was present in relatively very small amounts and approximately equal to those observed in pattern (a). It could be seen that the metabolite X_7 was excreted in greater amounts than when cortisone alone was administered but in lesser amounts than when both cortisone and a different ACTH preparation were given.

No characterization studies were carried out on the alpha-ketolic material designated as X_8 on the 24 hour chromatogram. However, one could see that the amounts of the $C_{21}O_4$ corticosteroids present in pattern (c) were approximately the same as when cortisone was administered and very significantly less than when both cortisone and another ACTH preparation were given together.

In summary, the paper chromatographic findings indicated that on cortisone administration substantial amounts of the biologically active $C_{21}O_5$ corticosteroids and their metabolites were excreted. At the same time essentially normal levels of the $C_{21}O_4$ corticosteroids appeared in the urine. The benzene-formamide and toluene-propylene glycol papergram scanning patterns showed surprisingly high urinary excretion of both $C_{21}O_5$ and $C_{21}O_4$ alpha-ketolic steroids when both cortisone and ACTH were administered. Pattern (c) illustrated a considerably lesser response on the administration of another ACTH preparation, alone, when evaluated by increase of corticosteroid excretion.

Case W.R.

The results on this patient with pronounced panhypopituitarism are shown on Figure 9. The scanning pattern of only 1 chromatogram that of the corticosteroids ranging in polarity from tetrahydro-F to compound E is shown. The free and enzyme hydrolysed fractions were chromatographed separately. The compounds found in each of the fractions are listed in Table VIII. The concentration of material applied for the free fraction (a) was 6.0 hours urine aliquot per cm. width of strip, i.e. 5 times that used for the conjugated fraction (b) which was 1.2 hours per cm.

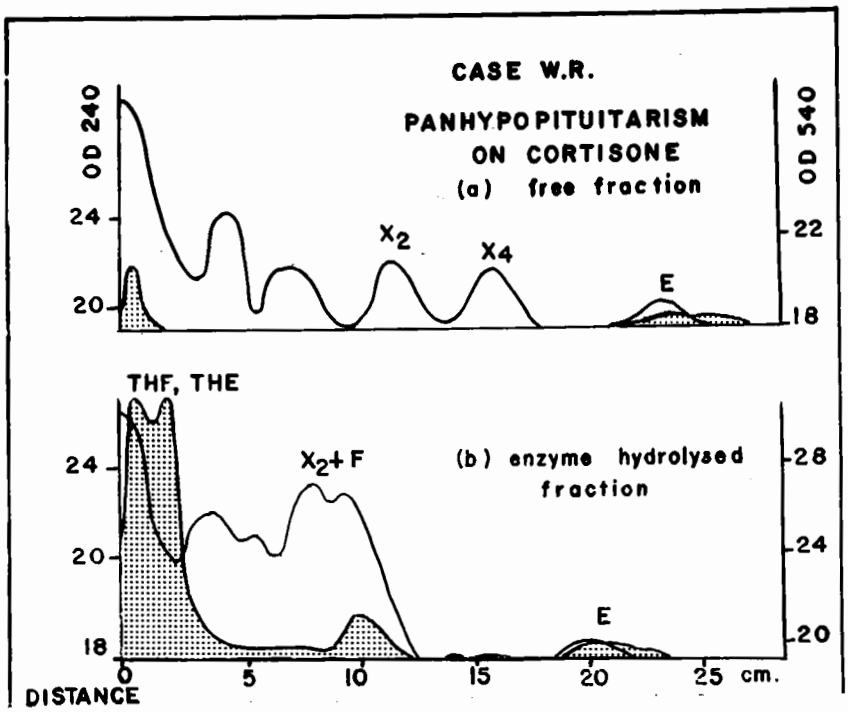
The total Porter-Silber values were very low,0.17 mgm. per 24 hours, when the patient was without steroid therapy. The pattern, which was obtained during cortisone administration, illustrated, therefore, essentially the metabolites of the administered cortisone.

The free fraction

A study of the chromatogram showed that despite the high concentration applied, there were only negligible amounts of the reduced metabolites tetrahydro-F and tetrahydro-E.

There could be seen a series of UV light absorbing, non-alpha-ketolic compounds. The metabolite found in the region in which hydrocortisone was characteristically present was designated as X_2. The regularly occurring metabolite, compound X_4 could be

FIGURE 9.



The scanning patterns of the urinary corticosteroids ranging in polarity from compounds THF to E for case W.R., a patient with hypopituitarism on cortisone therapy with the (a) free and (b) enzyme hydrolysed fractions being chromatographed separately. Concentration applied was 6.0 hour urine aliquot per cm. for the free fraction and 1.2 hour for the enzyme hydrolysed fraction.

Characterization studies carried out on corticosteroids isolated from urine.

TABLE VIII

Hypopituitarism, on cortisone administration, Case W.R.

Fraction	Compound	UV	BT
Free (a)	X_2	+	-
	X_4	+	-
	E	+	+
Enzyme hydrolysed (b)	THF	-	+
	THE	-	+
	X_2 + F	+ ,+	-,+
	E	+	+

seen in approximately the same amounts below X_2.

Hydrocortisone could not at all be detected upon the chromatogram while only about one-fifth of the cortisone excreted was recovered in the free fraction.

The enzyme hydrolysed fraction

By far the bulk of the compounds tetrahydro-F and tetrahydro-E was recovered in this fraction. Relatively very small amounts of hydrocortisone could be seen. The significant difference in the amount of UV light absorption and BT reduction at the region of hydrocortisone indicated that it was present as a mixture with the UV light absorbing, non-alpha-ketolic metabolite, compound X_2.

There were no traces of X_4 to be seen in the pattern while the amounts of cortisone were somewhat less than those of hydrocortisone. Taking concentration into account the results indicated that about four-fifths of the cortisone were recovered in the enzyme-hydrolysed fraction.

There were only negligible amounts of $C_{21}O_4$ corticosteroids present on the 24 hour chromatogram. For this reason the scanning patterns are expressly not shown.

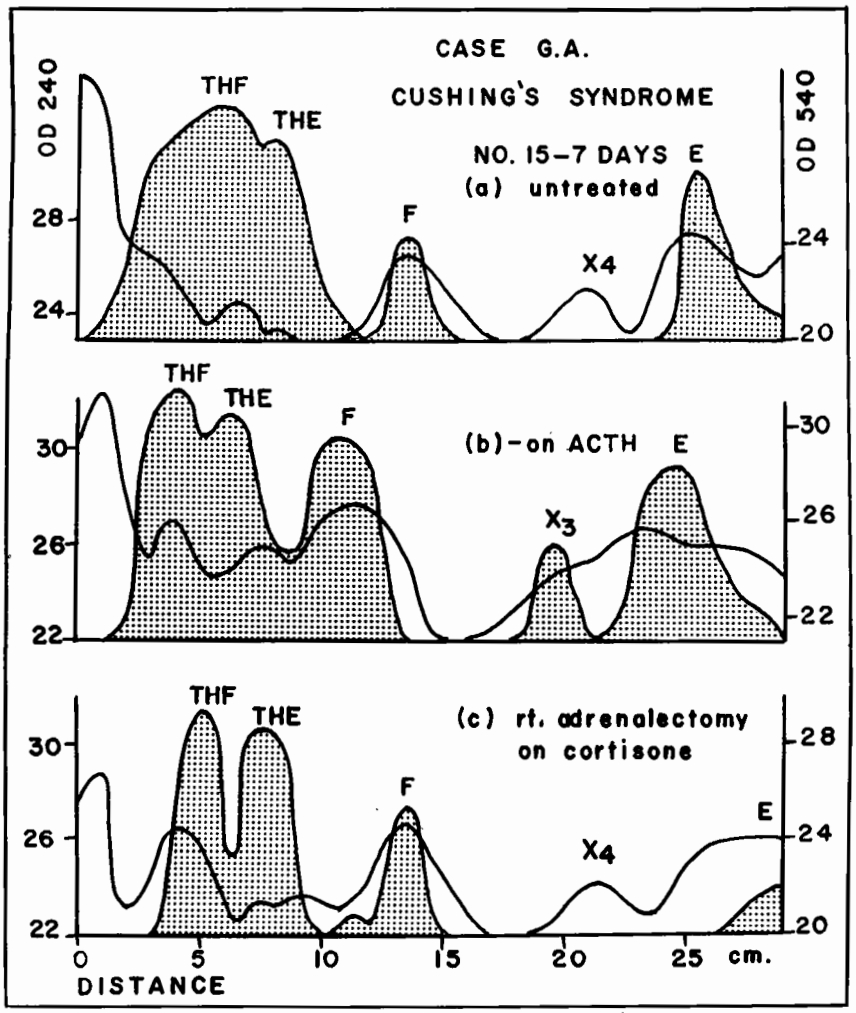
C. Patients with adrenal cortical hyperfunction

Case G.A.

The scanning patterns shown for case G.A. in Figures 10 to 12 are (a) of the untreated Cushing syndrome state, (b) on ACTH and (c) after a right adrenalectomy, when receiving 200 mgm. of cortisone per day. The corticosteroids are distributed over the 3 chromatograms in the following way. Those ranging in polarity from tetrahydro-F to compound E are shown on the 7 day chromatogram, Figure 10, while the corticosteroids shown on the 3 day chromatogram, Figure 11, mainly the X_8 complex, are derived from the overflow from the 7 day papergram. In turn the pattern on the 24 hour chromatogram, Figure 12, is that of the corticosteroids which have overflowed off the 3 day chromatogram. A 0.8 hours urine aliquot per cm. was applied thus allowing easy comparison of the relative amounts of the various corticosteroids with those of the normal subjects which were applied with the same concentration.

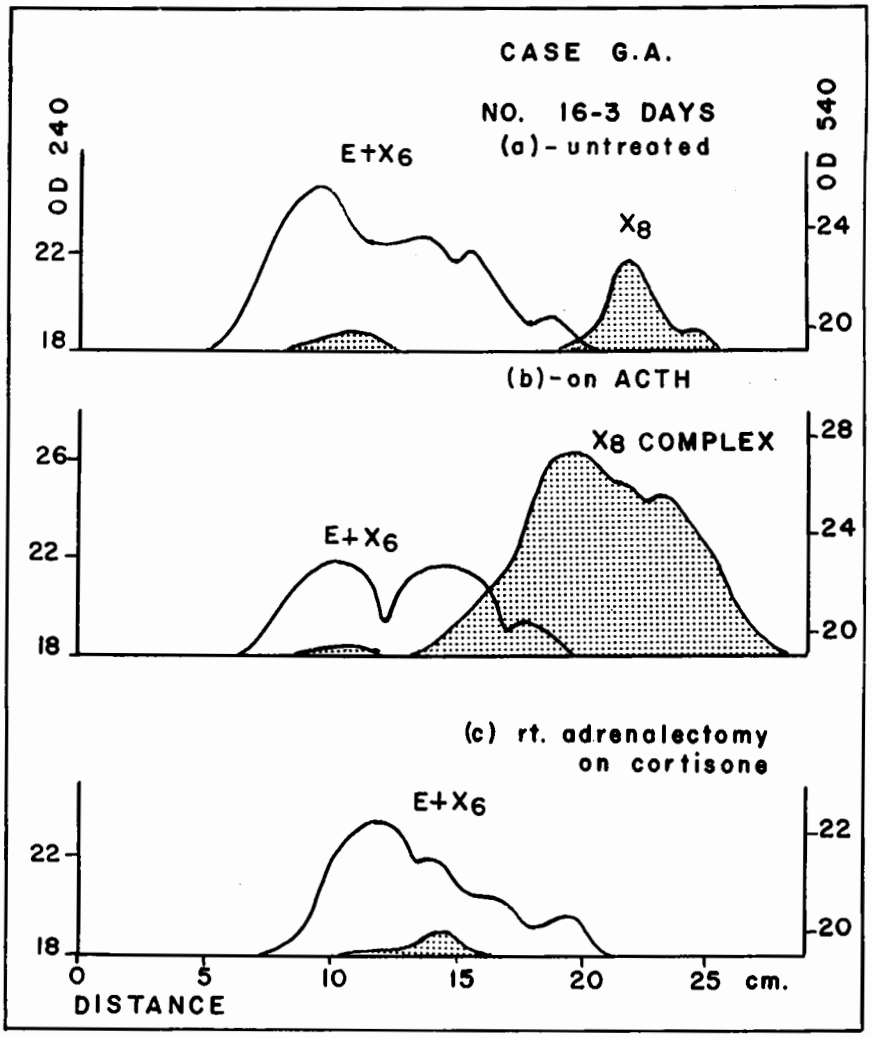
The three urinary corticosteroid patterns are presented separately. However, comparisons of the patterns are made throughout the description.

FIGURE 10.



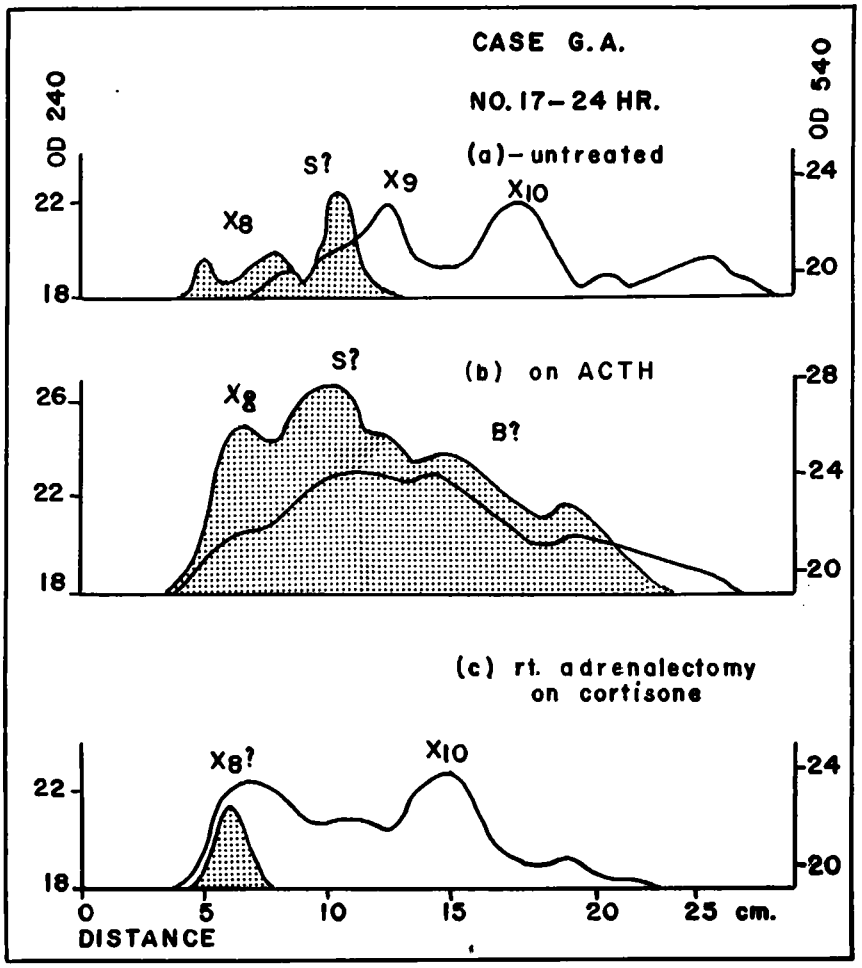
The scanning patterns of the urinary corticosteroids ranging in polarity from compounds THF to E, on the 7 day chromatogram, for case G.A., a patient with Cushing's syndrome in (a) the untreated disease state, (b) on ACTH administration and (c) after right adrenalectomy when receiving 200 mgm. of cortisone per day.
Concentration applied was 0.8 hour urine aliquot per cm.

FIGURE 11.



The scanning patterns of the urinary corticosteroids ranging in polarity from compound E to below compound THS, on the 3 day chromatogram, for case G.A., a patient with Cushing's syndrome in (a) the untreated disease state, (b) on ACTH administration and (c) after right adrenalectomy when receiving 200 mgm. of cortisone per day.
Concentration applied was 0.8 hour urine aliquot per cm.

FIGURE 12.



The scanning patterns of the urinary corticosteroids ranging in polarity from below compound THS to below compound B, on the 24 hour chromatogram, for case G.A. in (a) the untreated disease state, (b) on ACTH administration and (c) after right adrenalectomy when receiving 200 mgm. of cortisone per day. Concentration applied was 0.8 hour urine aliquot per cm.

Characterization studies carried out on corticosteroids isolated from urine

TABLE IX

Untreated Cushing's syndrome, Case G.A., pattern (a)

Compound	Source chrom. no.	eluted dist. cm.	UV	BT	PS	mgm/24 hr.	Mobility of compounds free
THF	15	1-7	-	+		18.40	runs with THF
THE	"	7-10	-	+			runs with THE
F	"	12-15	+	+		0.75	
X_4	"	19-23	+	-			
E	"	23-end	+	+		1.89	runs with E
(E) + X_6	16	8-12	+	+			
X_8	"	19-26	-	+	-		
X_8, S?	17	3-13	-, +	+, +		?	1) UV-BT+, slower than S 2) UV+BT+, runs with S
X_9	"		+	-			
X_{10}	"		+	-			

Characterization studies carried out on corticosteroids isolated from urine

TABLE X

Cushing's syndrome, on ACTH administration, Case G.A., pattern (b)

Compound	Source chrom. no.	eluted dist. cm.	UV	BT	PS	mgm /24 hr	Mobility of compound free	acetate
THF	15	0-5.5	-	+		16.65		
THE	"	5.5-9.0	-	+				
F	"	9.0-14.0	+	+		8.0		
X_3	"	18-22.5	+	+	+			UV-BT+, runs with solvent front
E	"	22.5-end	+	+	+		runs with E	
(E) + X_6	16	9-12	+	-				
*1 X_8	"	14-29	-	+	+			
X_8,B?,S?	17	4-24	-,+,+	+,+,+	+		1) UV-BT+slower than S *2 2) UV?BT+runs with S *2 3) UV+BT+runs with B *3	
X_{10}	"		+	-				

*1 Maxima of H_2SO_4 chromogen spectra were 310,430-440 may be mixture of THS and THB

*2 Maxima of H_2SO_4 chromogen spectra were 310,410 - may be mixture of compounds allo-THB and S

*3 May be compound B

Characterization studies carried out on corticosteroids isolated from urine

TABLE XI

On cortisone administration following right adrenalectomy for Cushing's syndrome, Case G.A., pattern (c)

Compound	Source chrom. no.	eluted dist., cm.	UV	BT	PS	mgm/24 hour	Maxima of H_2SO_4 chromogen spectra unknown/standard
THF	15	3-6	-	+		3.18	
THE	"	6-10	-	+			
F	"	10-16	+	+		0.57	$F = \frac{285,390,460-470}{285,390,460-470}$
X_4	"	19-23.5	+	-			
E	"	26-end	+	+	+		$E = \frac{285,343,420}{285,343,415}$
(E) + X_6	16	10-17	+	-			
X_8?	17	4-8	+	+	+		
X_{10}	"		+	-			

The scanning patterns of case G.A. in the untreated state of Cushing's syndrome are shown in Figures 10 to 12 (a). The results of characterization studies are included in Table IX.

Tetrahydro-F and tetrahydro-E were present in great amounts and together they formed the bulk of the alpha-ketolic steroids excreted in the urine. Porter-Silber estimation on aliquots of their combined eluates was 18.40 mgm. per 24 hours.

The most distinguishing feature of this pattern was the very high amount of cortisone excreted. Significant amounts of hydrocortisone were also present but in considerably lesser quantities than cortisone. Compounds F and E were estimated to be excreted at the rate of 0.75 and 1.89 mgm. per 24 hours respectively.

The metabolite X_4 was present in relatively small amounts.

The compound X_6 appeared on the 3 day papergram, Figure 11, in the region of cortisone but had no reducing properties and could be separated from cortisone only by longer development in toluene-propylene glycol.

One can see in Figures 11 and 12 that in comparison with the compounds E, THF and THE relatively insignificant amounts of X_8 and less polar $C_{21}O_4$ corticosteroids were present. The X_8-S complex subdivided on rechromatography into two well distinguished spots. The slower one designated in the pattern

with X_8 in a position slightly above compound S was only BT reducing and probably included compounds tetrahydro-B and tetrahydro-A since it formed no color in the Porter-Silber test. The faster material was located in the position of compound S. Its identity was doubtful because no definite Porter-Silber reaction could be obtained.

The characteristically occurring urinary metabolites X_9 and X_{10} could be seen on the 24 hour papergram, Figure 12, in substantial amounts while there was somewhat less UV absorption at the region of X_{11}.

The urinary corticosteroid pattern of case G.A. on ACTH administration is shown in Figures 10 to 12, (b) and the results of characterization studies are given in Table X.

The metabolites tetrahydro-F and tetrahydro-E were excreted in large amounts. However, the scanning pattern gives an indication and the Porter-Silber estimations show that on ACTH administration these combined compounds actually decreased slightly from 18.40 to 16.65 mgm. per 24 hours. It is also apparent that tetrahydro-F and tetrahydro-E were not the major components of the pattern as they were in that of the untreated disease state.

On the other hand, the biologically active corticosteroids, compounds F and E were excreted in relatively very

great amounts. Hydrocortisone was estimated to be excreted at the rate of 8.0 mgm. per 24 hours. When one compares patterns (a) and (b) it is apparent that when ACTH was administered the increment of increase in the excretion of hydrocortisone was considerably greater than that of cortisone.

In the region in which the metabolite X_4 is characteristically found there appeared on ACTH administration a compound designated as X_3 on the pattern. It was UV light absorbing, BT reducing, Porter-Silber positive and apparently disappeared after withdrawal of ACTH. On acetylation and rechromatography it was shown to be BT reducing but not UV light absorbing material with an increase in R_F corresponding to that of a diacetate. Thus the UV light absorption seen in Figure 10 was apparently not a property of X_3 but contributed from X_4 which clearly occurred in the other two patterns studied.

The metabolite X_6, Figure 11, was present in approximately the same amounts as in pattern (a).

On the administration of ACTH there was a very great increase in the amounts of the X_8 complex and less polar $C_{21}O_4$ corticosteroids excreted. The results of characterization studies, Table X, indicated that the unidentified X_8 may have consisted largely of tetrahydro-S and tetrahydro-B. The former compound

would have accounted for the positive Porter-Silber test obtained in this region while the presence of tetrahydro-B might have been considered because of chromatographic behaviour and H_2SO_4 chromogen spectrum. The alpha-ketolic material on the 24 hour papergram was rerun in mixed chromatograms. These procedures revealed 3 components, one more polar than compound S which behaved like some additional tetrahydro-B that had run off the 3 day papergram. The faster compound was of the same mobility as compound S and gave a strong BT reducing test and only weak absorption of UV light. These facts suggested together with the H_2SO_4 chromogen spectrum, the presence of mainly allo-tetrahydro-B and some compound S. The fastest component gave color tests like corticosterone and since it did not separate from it in the mixed chromatogram it may have been compound B.

The metabolites X_9 and X_{10} could not be clearly seen because of the large amount of alpha-ketolic material in the same region, Figure 12. As in pattern (a) there were only insignificant amounts of UV light absorbing material at the region of X_{11}.

The urinary corticosteroid pattern of case G.A. after

right adrenalectomy when receiving 200 mgm. of cortisone per day is illustrated in Figures 10 to 12. The results of characterization studies are given in Table XI.

The reduced metabolites tetrahydro-F and tetrahydro-E were excreted in substantial amounts (3.18 mgm. per 24 hours) and were by far the major components of the pattern. However, it is apparent on comparison with patterns (a) and (b) that despite the high cortisone dosage administered there was a definite decrease in the urinary output of these compounds after right adrenalectomy.

On the other hand, despite the decrease in the excretion of tetrahydro-F and tetrahydro-E the output of hydrocortisone was still considerable (0.57 mgm. per 24 hours) while somewhat lesser quantities of cortisone were also excreted. It can be seen in Figure 10, that after right adrenalectomy the amount of hydrocortisone excreted was almost as great as in the untreated disease state while the output of cortisone was very much decreased despite the high cortisone dosage administered.

There was no change in the quantitative pattern of the metabolites X_4 and X_6.

The outstanding characteristic of the pattern after right adrenalectomy was the lack of X_8 in the region below cortisone in contrast to the significant amount observed in the untreated

disease state and the very great amount excreted on the administration of ACTH.

The small quantity of unidentified alpha-ketolic material on the 24 hour chromatogram, Figure 12, also gave a positive Porter-Silber test. It was probably not compound S since no amount of its metabolite, tetrahydro-S was found in the region below cortisone on the 3 day chromatogram.

The scanning patterns indicated that the metabolites X_9 and X_{10} were excreted at a steady rate in case G.A. independent of the physiological state studied. On all patterns there was insignificant UV light absorption at the region of X_{11}.

In summary, the chromatographic patterns of case G.A. are of interest because they showed a type of Cushing's syndrome in which the urinary output of compounds F and E was abnormally high while the rate of excretion of the $C_{21}O_4$ corticosteroids was not increased to the same extent. The administration of ACTH had a marked effect in altering the quantitative and qualitative pattern of both the $C_{21}O_5$ and $C_{21}O_4$ corticosteroids excreted in the urine and resulted in the appearance of a hitherto undetected metabolite, X_3, between the regions of hydrocortisone and cortisone on the papergram. The pattern after right adrenalectomy was essentially that of the metabolites of

the administered cortisone but unusual in that no more polar, $C_{21}O_4$ corticosteroids (the X_8 complex) was present.

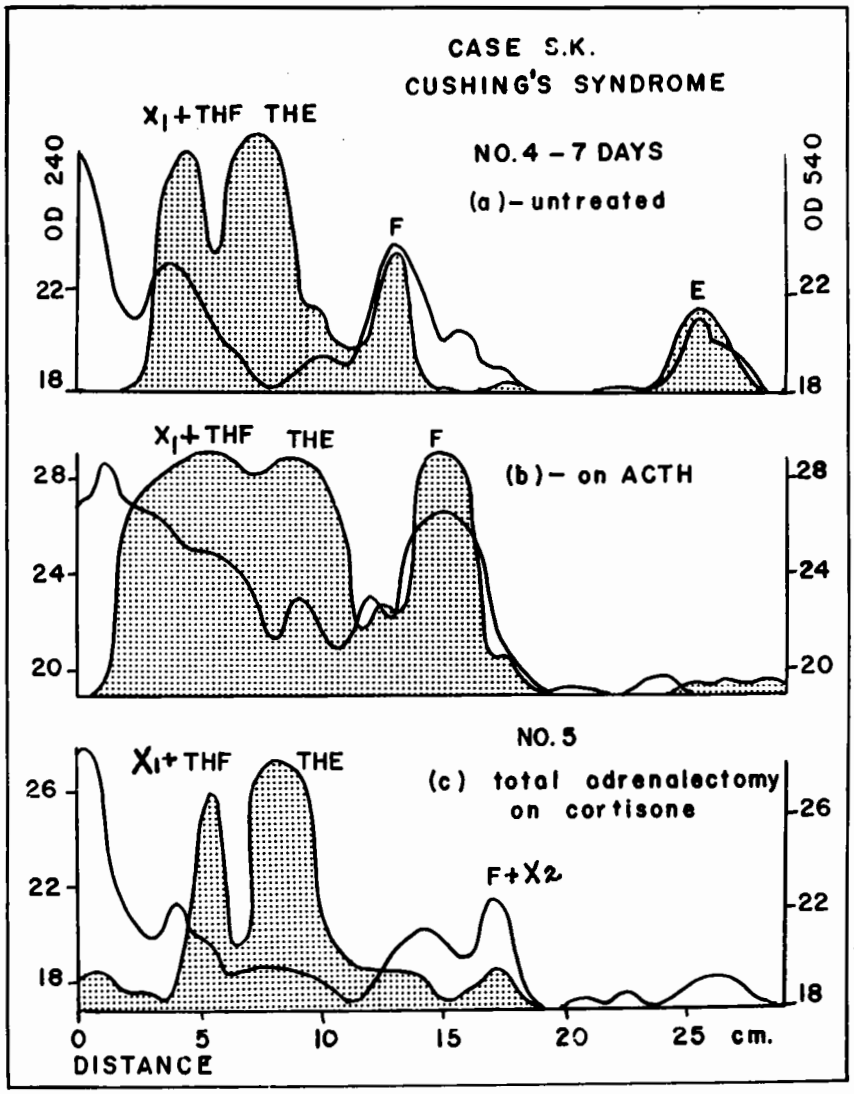
Case S.K.

This patient was a case of Cushing's syndrome who was studied in the untreated disease state, pattern (a), during ACTH administration, pattern (b) and after total adrenalectomy when receiving 50 mgm. of cortisone per day.

Prior to a description of the results obtained with case S.K. it must be recalled that the chromatographic procedure was somewhat different from that used for case G.A. but identical to that used for the normal subjects. Briefly, different aliquots of the $C_{21}O_5$ corticosteroids ranging in polarity from tetrahdyro-F to that of cortisone were chromatographed on both the 7 and 3 day chromatograms, Figures 13 and 14 and in addition part of X_8 was included on the latter. However, the picture of the corticosteroids ranging from tetrahydro-F to hydrocortisone was omitted from the scanning pattern on the 3 day chromatogram in order to facilitate comparison with that of case G.A. also, the pattern of these metabolites was already shown on the 7 day chromatogram. In addition, the 24 hour papergrams of cases S.K. and G.A. differ in that all the $C_{21}O_4$ corticosteroids (including all of X_8) present in each extract are shown for S.K., whereas in case G.A. the 24 hour chromatogram merely included any X_8 that had overflowed from the 3 day papergram plus any less polar $C_{21}O_4$ corticosteroids present in the extract.

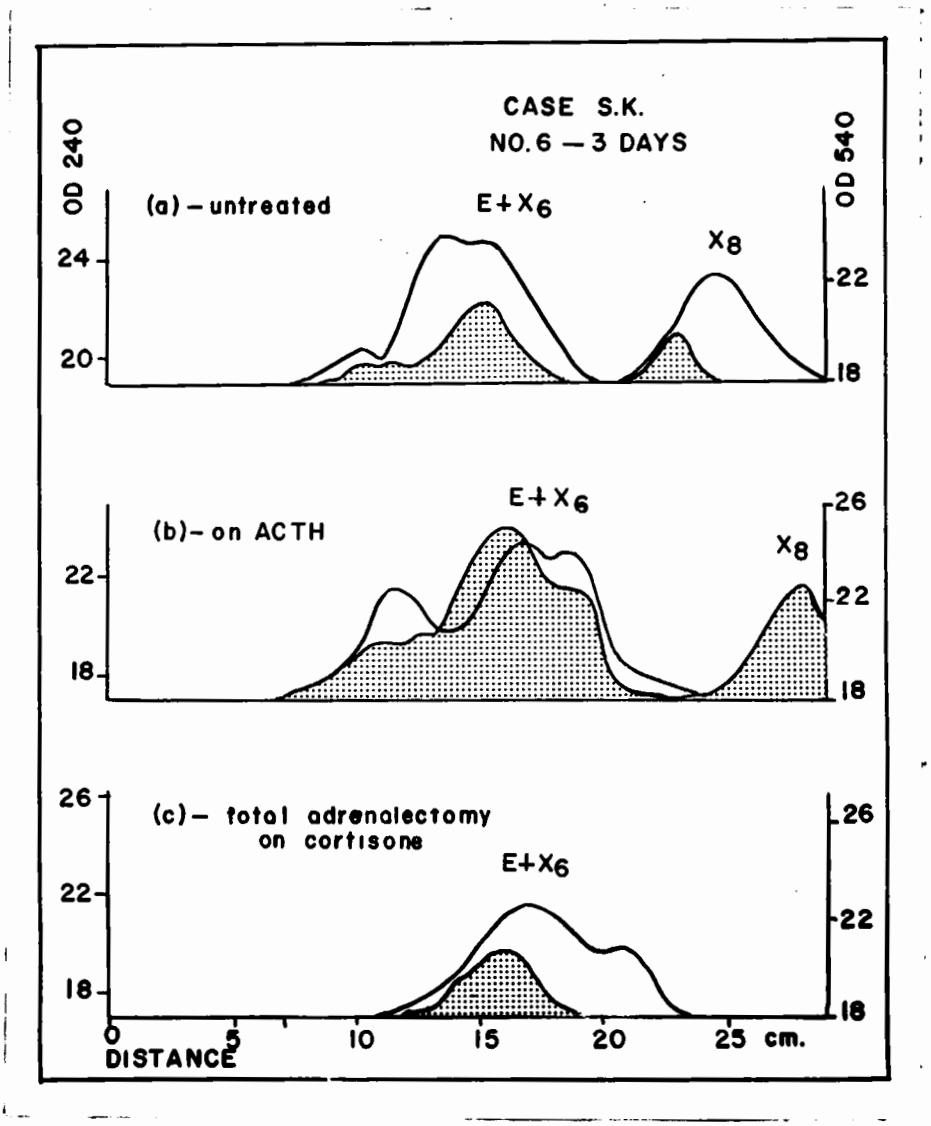
Nevertheless, the relative amounts of the various

FIGURE 13.



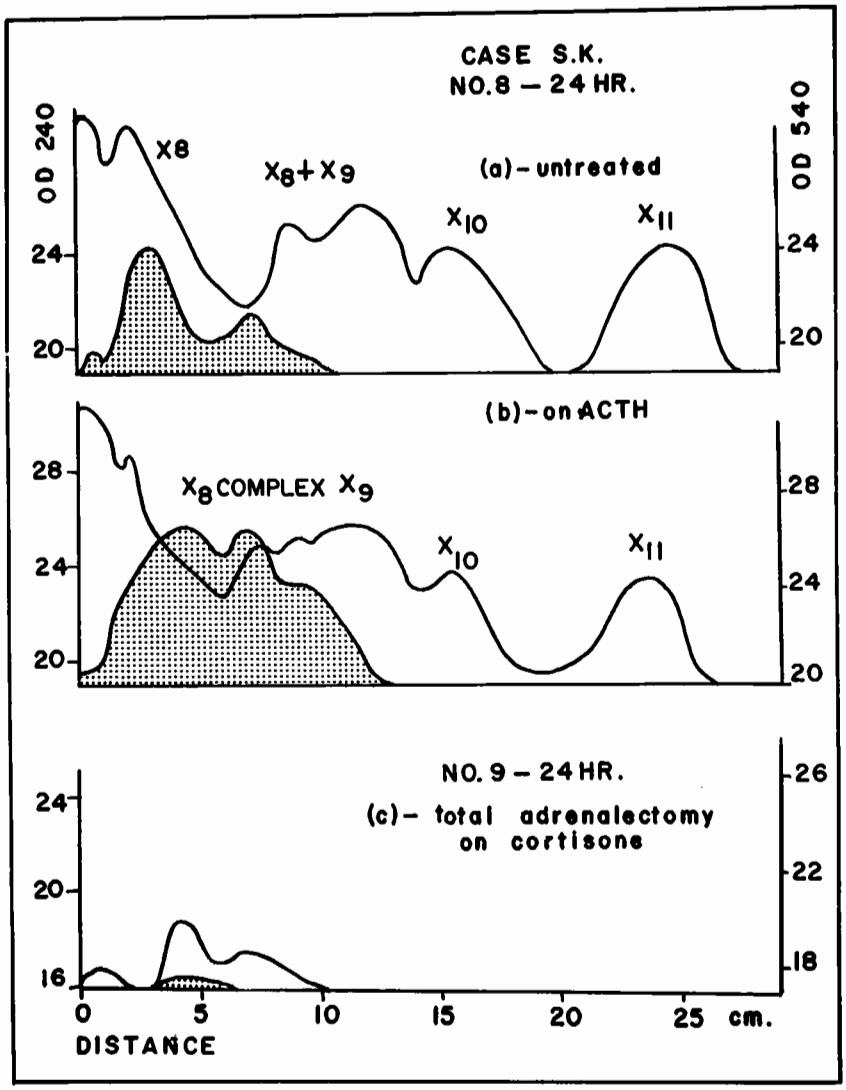
The scanning patterns of the urinary corticosteroids ranging in polarity from compounds THF to below E, on the 7 day chromatogram for case S.K. a patient with Cushing's syndrome in (a) the untreated disease state, (b) on ACTH administration and (c) after total adrenalectomy when receiving 50 mgm. of cortisone per day. Concentration applied was 0.8 hour urine aliquot per cm.

FIGURE 14.



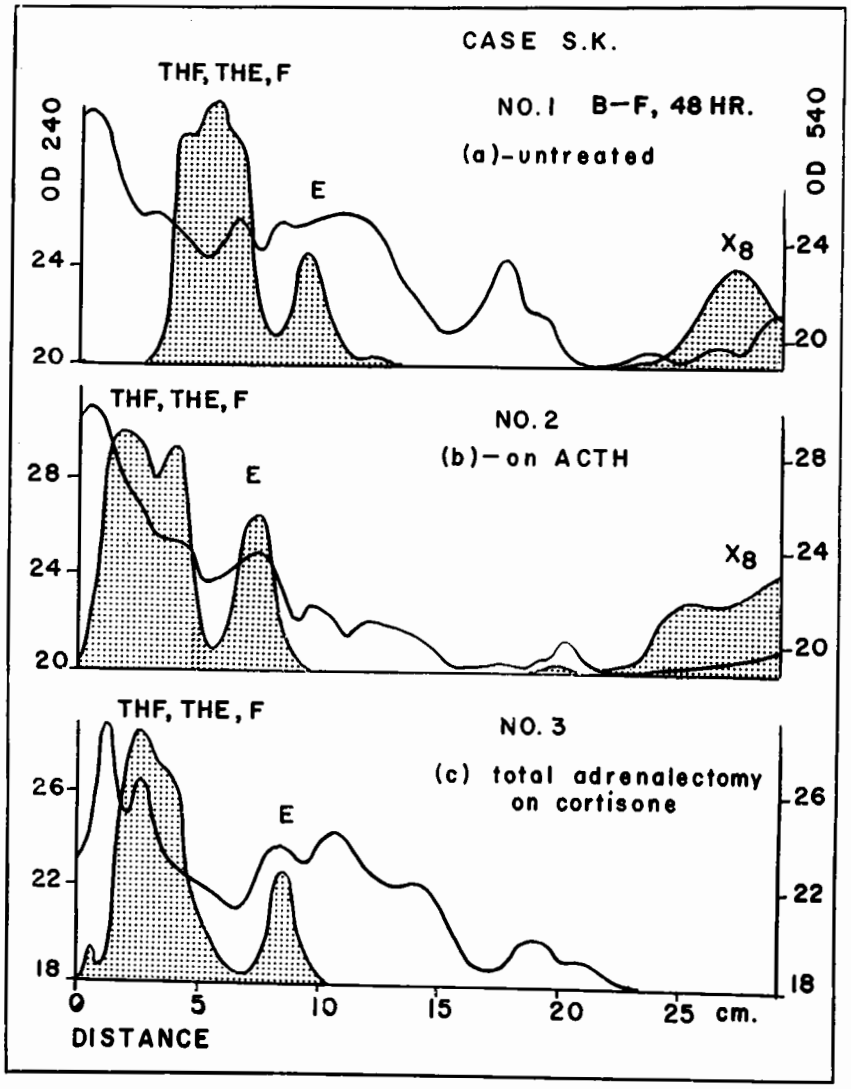
The scanning patterns of the urinary corticosteroids ranging in polarity from below compound F to below compound THS, on the 3 day chromatogram for case S.K., a patient with Cushing's syndrome in (a) the untreated disease state, (b) on ACTH administration and (c) after total adrenalectomy when receiving 50 mgm. of cortisone per day.
Concentration applied was 0.8 hour urine aliquot per cm.
N.B. UV light absorbing and BT reducing peaks have been mistakenly reversed at the position of X_8 in pattern (a).

FIGURE 15.



The scanning patterns of the $C_{21}O_4$ urinary corticosteroids ranging in polarity from below compound E to below compound B on the 24 hour chromatogram, for case S.K. a patient with Cushing's syndrome in (a) the untreated disease state (b) on ACTH administration and (c) after total adrenalectomy when receiving 50 mgm. of cortisone per day.
Concentration applied was 0.8 hour urine aliquot per cm.

FIGURE 16.



The scanning patterns of the urinary corticosteroids ranging in polarity from compound THF to below compound THS, on the initial benzene-formamide chromatograms for case S.K., a patient with Cushing's syndrome in (a) the untreated disease state (b) on ACTH administration and (c) after total adrenalectomy when receiving 50 mgm. of cortisone per day.
Concentration applied was 0.8 hour urine aliquot per cm.

Characterization studies carried out on corticosteroids isolated from urine

TABLE XII

Untreated Cushing's syndrome, case S.K., pattern (a)

Compound	Source chrom. no.	eluted dist.cm.	UV	BT	PS	Mobility of compounds free
THF	4	2-5.5	-	+	+	
THE	"	5.5-10	-	+	+	
F	"	11-14	+	+	+	
E+X_6	6	13-17	+	+	+	
X_8, X_8 +X_9	8	1-11	+	+	+	1) UV-BT+ slower than DHE 2) UV+BT- runs with DHE 3) UV-BT+runs with THA
X_{10}	8		+	-		
X_{11}	8		+	-		

Characterization studies carried out on corticosteroids isolated from urine

TABLE XIII

Cushing's syndrome, on ACTH administration, Case S.K., pattern(b)

Compound	Source chrom. no.	eluted dist. cm.	UV	BT	PS	Mobility of compounds free
THF	4	0-7	-	+	+	
THE	"	7-12	-	+	+	
F	"	12-19	+	+	+	runs with F
E+X_6	6	14-22	+	+	+	1) UV-BT+ slower than E* 2) UV+ BT+ runs with E
X_8+X_9	8	1-13	-, +	+, +	+	1) UV-BT+ slower than DHE 2) UV+BT+ slower than DHE 3) UV+BT- runs with DHE 4) UV-BT+ runs with THA
X_{10}	"		+	-		
X_{11}	"		+	-		

* This indicates the presence of the compound X_3 between hydrocortisone and cortisone

Characterization studies carried out on corticosteroids isolated from urine

TABLE XIV

On cortisone administration following total adrenalectomy for Cushing's syndrome, Case S.K., pattern (c)

Compound	Source chrom. no.	eluted dist. cm.	UV	BT	PS
THF	5	3-6	-	+	+
THE	"	6-11	-	+	+
F	"	15-18	+	+	+
$E+X_6$ *	7	13-19	+	+	+

* Complete absence of X_8 belowcompound E; no UV BT- compounds at regions of X_9, X_{10} and X_{11}.

corticosteroids which separated in each of the chromatographic patterns of cases S.K., G.A. and the normal subjects could be easily compared as the same concentration, 0.8 hours urine aliquot per cm. width of strip, was used throughout.

The three urinary corticosteroid patterns are presented separately. However comparisons of the patterns are made throughout the description.

The urinary corticosteroid pattern of case S.K. in the untreated state of Cushing's syndrome is illustrated in Figures 13 to 16, (a). The results of characterization studies are included in Table XII.

The metabolites tetrahydro-F and tetrahydro-E were excreted in increased amounts and formed the bulk of the alpha-ketolic material present in the urinary corticosteroid pattern.

The output of the biologically active corticosteroids, compounds F and E was correspondingly increased. There were no significant differences in the excretory rates of these compounds. (Cortisone reappeared in the pattern on the 3 day chromatogram, Figure 14 and was present as a mixture with the UV light absorbing non-alpha-ketolic metabolite, X_6).

One can see in Figure 15 that in comparison with the compounds F, E and their metabolites only small amounts of X_8

and less polar $C_{21}O_4$ corticosteroids were excreted. The alpha-ketolic material on the 24 hour papergram was rechromatographed in benzene-formamide. There was a separation into at least two BT reducing compounds with no UV light absorption and with the same polarity as standard compounds DHE and THA. Since this material was quantitatively Porter-Silber positive it may have included the metabolite tetrahydro-S which was not, however, identified to be present.

The series of UV light absorbing metabolites X_9, X_{10} and X_{11} were present in considerable amount.

The scanning patterns of case S.K. on ACTH administration are shown in Figures 13 to 16, (b) while the results of characterization studies are included in Table XIII.

On the administration of ACTH the urinary output of the metabolites tetrahydro-F and tetrahydro-E was greatly increased above that observed in the untreated disease state.

There was a correspondingly great increase in the excretion of the biologically active corticosteroids, compounds F and E.

Although the presence of X_3 had not been designated on the scanning pattern of the 3 day chromatogram there was

certainly an indication on the pattern, Figure 14, that more than 1 BT reducing steroid was present at the region of cortisone. Upon rechromatography there was a true separation into two compounds, one was cortisone and the other a more polar BT reducing compound with no UV light absorption which was probably identical to the metabolite X_3 of pattern (b) in case G.A., Figure 10.

It is apparent that ACTH administration did effect an increase in the urinary output of the X_8 complex and less polar $C_{21}O_4$ corticosteroids when one compares patterns (a) and (b), Figure 15. However, the increment in the increase of the $C_{21}O_4$ corticosteroids was considerably less than that observed for the compounds THF, THE and F. Upon rechromatography of X_8 there was a separation into at least 4 different compounds, three of which reduced BT.

The pattern of the metabolites X_9, X_{10} and X_{11} remained unchanged on the administration of ACTH.

The urinary corticosteroid pattern of case S.K. after total adrenalectomy when receiving 50 mgm. of cortisone

per day is illustrated in Figures 13 to 16, (c), while the results of characterization studies are included in Table XIV.

The metabolite tetrahydro-E was excreted in substantial amounts but the urinary output of tetrahydro-F was considerably decreased. A comparison of the patterns shows this very well.

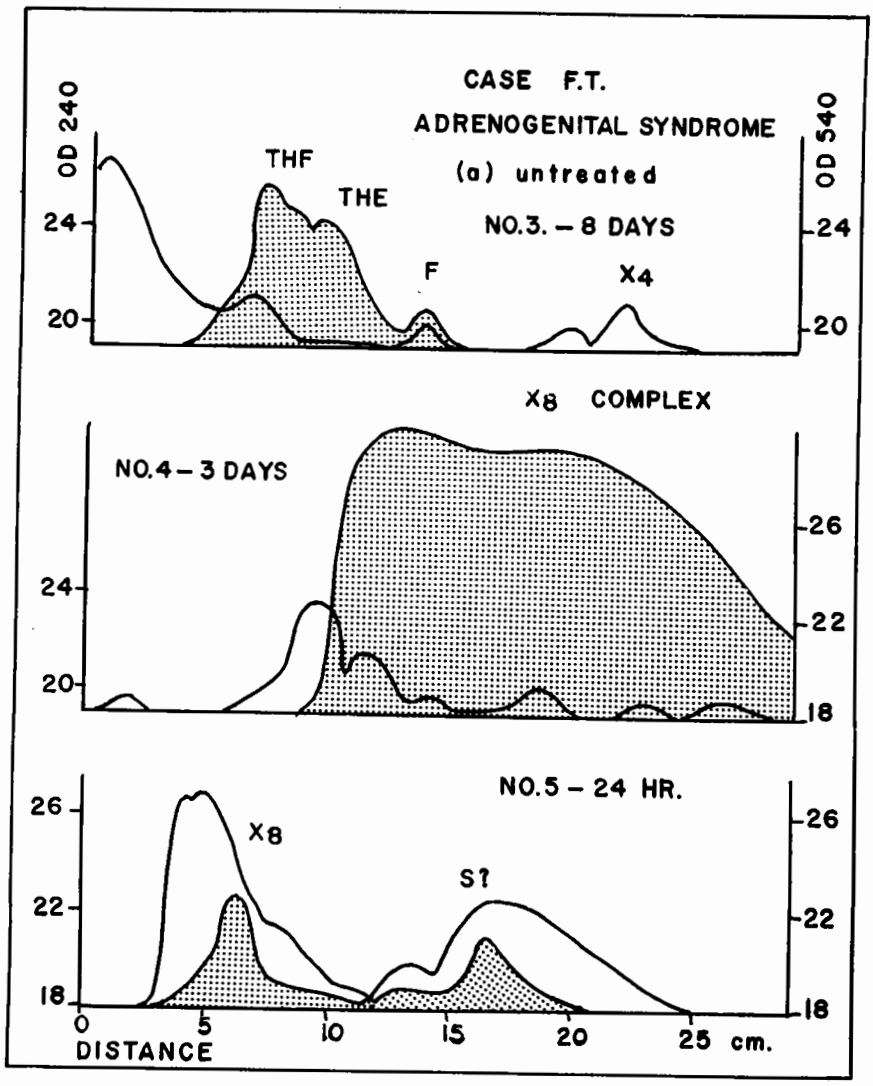
The excretion of hydrocortisone was lower than that observed in the untreated disease state and much lower than that observed on ACTH administration. The urinary output of cortisone was not decreased to the same extent. There was also an indication that somewhat lesser quantities of X_6 were excreted, Figure 14.

After total adrenalectomy, when the patient received cortisone therapy there was a complete lack of X_8 and less polar $C_{21}O_4$ compounds in the urinary corticosteroid pattern. This finding on the toluene-propylene glycol chromatogram is also illustrated in the scanning patterns of the initial benzene-formamide papergrams, Figure 16. As can be seen slightly greater amounts of X_8 were present in the pattern after ACTH administration than in that of the untreated disease state. However, there was no X_8 in the pattern after total adrenalectomy.

The metabolites, X_9, X_{10}, and X_{11}, which had been regularly occurring in all the patterns thus far described were absent.

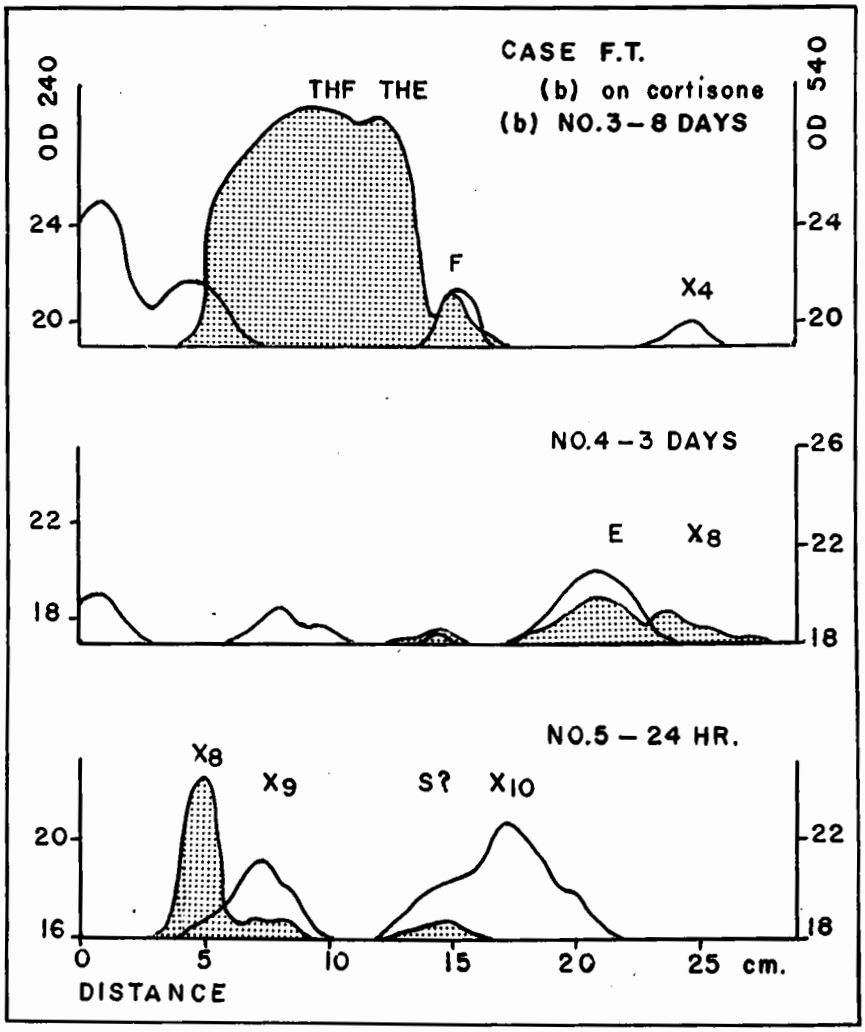
In summary, the results showed that in this patient with Cushing's syndrome there was abnormally high excretion of the biologically active corticosteroids, compounds F and E and of their metabolites while the urinary output of the $C_{21}O_4$ corticosteroids was not increased to the same extent. The response to ACTH was mainly restricted to the still greater urinary excretion of the $C_{21}O_5$ corticosteroids and their metabolites. Since extract (c) was obtained after total adrenalectomy the pattern was only that of the metabolites of the exogenous cortisone, showing these to include tetrahydro-F, tetrahydro-E and compounds X_1, X_2 and X_6. The unusual feature of this pattern was the absence of $C_{21}O_4$ corticosteroids and the metabolites X_9, X_{10} and X_{11}.

FIGURE 17.



Case F.T. a patient studied in the untreated state of congenital adrenal hyperplasia and showing scanning patterns of the urinary corticosteroids ranging in polarity from compounds THF to below E on the 8 day chromatogram; from polarity below compound F to below compound THS on the 3 day chromatogram and from polarity below compound THS to below compound B on the 24 hour chromatogram. Concentration applied was 1.6 hour urine aliquot per cm.
N.B. UV light absorbing and BT reducing peaks have been mistakenly reversed at the position of X_8 on the 24 hour chromatogram.

FIGURE 18.



Case F.T., a patient with congenital adrenal hyperplasia (adrenogenital syndrome) studied on cortisone therapy and showing scanning patterns of the urinary corticosteroids ranging in polarity from compounds THF to below E on the 8 day chromatogram; from polarity below compound F to below compound THS on the 3 day chromatogram and from polarity below compound THS to below compound B on the 24 hour chromatogram.
Concentration applied was 1.6 hour urine aliquot per cm.

Characterization studies carried out on corticosteroids isolated from urine

TABLE XV

Untreated congenital adrenal hyperplasia, Case F.T., pattern (a)

Compound	Source chrom. no.	eluted dist. cm.	UV	BT	PS	Mobility of compound free	Mobility of compound acetate	Maxima of H_2SO_4 chromogen spectra unknown / standard
THF	3	3-12	-	+				
THE	"	"	-	+				
F	"	12-15	+	+				
X_4	"		+	-				
X_8	4	9-end	-	+	+	runs with THS	*moves with solvent front	320,410 / THS= 315,410
S?	5	15-23	+	+	+	1) UV+BT+runs with S 2) UV-BT+runs with B		

* $-X_8$ formed either a di- or polyacetate - maxima of H_2SO_4 chromogen spectra were 310, 430, (500)

TABLE XVI

Congenital adrenal hyperplasia, on cortisone administration, Case F.T., pattern (b)

Compound	Source chrom. no.	eluted dist.cm.	UV	BT	PS	Mobility of compounds free	acetate
THF	3	4-14	-	+			
THE	"	"	-	+			
F	"	14-16	+	+		runs with F	
X_4	"		+	-			
$E+X_8$	4	17-27	+,-	-		1) UV+BT+runs with E 2) UV-BT+faster than E*1	
X_8	5	3-9	-	+	+		1) UV-BT+slower than Sac 2) UV-BT+moves with solvent front *2
S?,X_{10}	"	13-20	+	+	-		

*1 Maxima of H_2SO_4 chromogen spectra were 315,420 - maxima of standard THS are 315,410

*2 " " H_2SO_4 " " " " 315,395

Case F.T.

This patient was a case of congenital adrenal hyperplasia who was studied in the untreated condition and during cortisone therapy. The scanning patterns of the corticosteroids ranging in polarity from tetrahydro-F to below compound F are shown on the 8 day chromatogram. The overflow of this latter paper was rechromatographed on the 24 hour run and shows the less polar components of X_8 and any other less polar $C_{21}O_4$ corticosteroids, if present. The concentration of material applied was 1.6 hours urine aliquot per cm. width of strip -i.e., double that used for the normal subjects and Cushing's syndrome cases.

The scanning patterns for case F.T. in the untreated disease state, (a), are shown in Figure 17. The results of characterization studies are given in Table XV.

The urinary metabolites tetrahydro-F and tetrahydro-E were identified in pattern (a). It is interesting to observe (especially when concentration is taken into account) that they were present in unusually small amounts.

Compound F was also identified to be present but in very small amounts. In fact, had a lower concentration been applied, hydrocortisone would not have been detectable on the scanning pattern. The regularly occurring metabolite X_4 was also present in small amounts.

However, the pattern of the medium polar corticosteroids shown on the 3 day chromatogram is strikingly different from any studied in this investigation. A large amount of alpha-ketolic steroid material completely covered the region of cortisone and X_8. A number of characterization studies have been carried out on this X_8 complex of pattern (a) which indicate that the substance may have been at least in greatest part, the reduced metabolite of compound S, tetrahydro-S. This was concluded on the basis of similar chromatographic behaviour in toluene-propylene glycol and benzene-formamide and similar absorbing maxima in concentrated H_2SO_4 as the authentic compound. The metabolite X_8 was also shown to be Porter-Silber positive and on acetylation there was observed an increase in R_F value corresponding to that of at least a diacetate. The absorbing maxima of the acetate in concentrated H_2SO_4 were 310,430,(500).

The results of characterization studies showed that in pattern (a) the alpha-ketolic material below X_8 on the 24 hour chromatogram consisted of UV light absorbing, BT reducing material with the same chromatographic behaviour as compound S in analytical runs and also consisted of a BT reducing compound with no UV light absorption and with the same chromatographic behaviour as compound B. Since this region gave a quantitatively positive Porter-Silber test and because of the very large amount

of a compound present which had been characterized to be its reduced derivative, tetrahydro-S, the UV light absorbing compound may have been compound S. However, its presence was only inferred and it was not conclusively proved to be compound S.

The scanning patterns for case F.T. on cortisone administration, (b) are shown in Figure 18. The results of any characterization studies are included in Table XVI.

On cortisone administration there was a striking change in the urinary corticosteroid pattern. The levels of tetrahydro-F and tetrahydro-E excreted in the urine were significantly increased over that observed in pattern (a). There was, however, only a slight increase in the urinary output of hydrocortisone and the amounts of X_4 present were approximately the same as in the untreated state. The greatest alteration was in the pattern of the medium polar corticosteroids showing almost a complete suppression of the excretion of a compound which was characterized to be tetrahydro-S and of other components of X_8 in the urine. Normal amounts of cortisone were excreted while the traces of X_8 below cortisone have been characterized to be tetrahydro-S by H_2SO_4 chromogen spectrum. The amount of alpha-ketolic material designated as compound S in the region of X_{10} was too little to permit characterization studies. However, since it did not give a positive Porter-Silber test, compound S was very likely not a component of this region.

In summary, the paper chromatographic findings showed that the urinary corticosteroid pattern of case F.T. in the untreated state of congenital adrenal hyperplasia was very different from that observed in the normal subjects or in patients with Cushing's disease. The X_8 complex (probably included in greatest part the metabolite tetrahydro-S) was by far the major corticosteroid in the urinary corticosteroid pattern. Subnormal amounts of hydrocortisone were excreted while the reduced metabolites of the $C_{21}O_5$ corticosteroids, tetrahydro-F and tetrahydro-E had become relatively minor urinary constituents. However, on the administration of cortisone this unusual corticosteroid pattern completely altered and became essentially normal. The metabolites, tetrahydro-F and tetrahydro-E, formed once more the bulk of the alpha-ketolic steroids excreted although the levels of hydrocortisone were not significantly increased. The excretion of X_8 had become almost completely suppressed and detectable amounts of cortisone were present on the chromatogram.

V. Discussion

The chromatographic patterns obtained in the normal subjects indicated that the male and female urinary corticosteroid patterns were essentially similar both qualitatively and quantitatively with respect to $C_{21}O_5$ steroid excretion. In both subjects the urinary excretion of the biologically active compound E was somewhat greater than that of compound F, which is probably related to the finding of substantially greater amounts of tetrahydro-E than of tetrahydro-F in the urine. However, the finding of only negligible amounts of X_8 and less polar corticosteroids in the female pattern studied, as compared to that of the male, should not according to previous experience in this laboratory be interpreted as a sex difference (88). Apparently, normal female urine ordinarily contains significant amounts of these compounds. The results obtained would seem to indicate that in the normal subjects there was greater adrenal cortical activity with respect to the secretion of the $C_{21}O_5$ corticosteroids such as compounds F and E while the secretion of compounds such as S, B or A was considerably less; thus leading to the urinary excretion of greater amounts of the reduced metabolites of hydrocortisone and cortisone as compared to those of the $C_{21}O_4$ corticosteroids.

Wilson et al. (112) and Dohan et al.(34) have made similar observations. Bush and Sandburg (34) have measured the levels

of the biologically active corticosteroids in the peripheral blood of normal subjects and have estimated the ratio of hydrocortisone to corticosterone to be, at the lowest, approximately 4 to 1.

In addition to the regularly occurring corticosteroids present on the patterns studied, i.e., compounds THF, THE, F and E, the compounds B, DHE and THS have been found by Zaffaroni (18), Schneider (92) and Touchstone (100) in normal urine.

The urinary corticosteroid pattern of case D.M. in the untreated state of hypopituitarism was qualitatively similar to the pattern of the normal male but the proportion of compound F and tetrahydro-F to compound E and tetrahydro-E was relatively greater. When one recalls the different concentrations applied on the papergrams it is apparent that all the metabolites were present in significantly lesser quantities. Nevertheless, the chromatographic findings might be interpreted to indicate that in this case of hypopituitarism the lowered adrenal cortical function was essentially of the same character as in the normal individual, that is, the adrenal cortex may have secreted greater amounts of the more polar biologically active corticosteroids such as compound F into the circulation as compared to those of com-

pounds S, B or A. The low glucose tolerance observed in this patient may have been related to the abnormally low output of the glucocorticoids, cortisone and hydrocortisone. However, there was no indication on the pattern for an explanation of the unusual finding of hypertension in this patient with hypopituitarism.

The patient did not respond to the administration of ACTH. Apart from the appearance of greater amounts of cortisone on the chromatogram, ACTH did not alter to any extent the urinary corticosteroid pattern when compared to that of the untreated disease state. The lack of response was probably due to the fact that the patient had been reciving cortisone for some time. This treatment might have lead to the further supression of adrenal cortical activity thus modifying the response to the ACTH which was administered soon after cortisone therapy was ceased. It is very likely that had ACTH administration been prolonged the response of the adrenals would have been more significant in time.

Other investigators have studied the urinary corticosteroid pattern of patients with severe panhypopituitarism and have found only negligible amounts of corticosteroids. Richardson et al. (84) have published the scanning pattern of one case in which higher concentrations of extract were applied but no such significant peaks of alpha-ketolic steroids could be detected as in the pattern of case D.M., a patient with only moderate hypo-

pituitarism. Gray (48) has studied the urinary corticosteroid pattern of patients with hypopituitarism before and after ACTH administration. In most cases a large increase in the urinary corticosteroid output was effected with ACTH, similar to that observed by Dohan et al. (34) and Mason et al. (67) in normal subjects. This is again in contrast to the finding in the patient, case D.M.

No control urinary corticosteroid pattern was obtained in case A.L., a patient with severe hypopituitarism, but the urinary output of endogenous 17-hydroxycorticosteroids was measured to be very low. Thus the pattern obtained on the administration of cortisone alone was essentially that of the metabolites of cortisone. Since the results of this investigation have shown that cortisone is not degraded to $C_{21}O_4$ corticosteroids in metabolism to any extent, it was of interest to observe the presence of essentially normal amounts of these steroids which were presumably of adrenal origin in the urinary corticosteroid pattern when cortisone was administered alone.

Before the results of the chromatographic patterns obtained on both cortisone and ACTH administration to this patient with hypopituitarism, case A.L., were described, it was pointed

out that any differences between this pattern and the one on cortisone administration alone should be attributed to the effects of the ACTH. The paper chromatographic results indicated surprisingly high urinary excretion of the $C_{21}O_5$ corticosteroids, their reduced metabolites and of the components of the X_8 complex which probably included the compound tetrahydro-S and less polar $C_{21}O_4$ corticosteroids. In fact, taking concentration into account, the urinary corticosteroid pattern on this combination of hormone therapy resembled very closely that of the untreated state of Cushing's syndrome in the patient, case G.A. The results might be interpreted to indicate that in this patient with severe hypopituitarism the adrenal cortical activity was greatly stimulated by the particular Nordic ACTH preparation administered, Acton, thus leading to the abnormally high secretion of both the $C_{21}O_5$ glucocorticoids such as compounds F and E and of the $C_{21}O_4$ biologically active corticosteroids such as compounds S and B. This marked response observed after both ACTH and cortisone administration was then similar to that studied by a number of investigators including Forsham (42) and Thorn (99) in patients with hypopituitarism after ACTH administration alone.

It is interesting to note that a quite different urinary corticosteroid pattern was studied in the patient, case A.L., after the administration of only the long-acting Nordic ACTH preparation,

Duracton. The paper chromatographic results indicated how varied the response to ACTH could be in a single individual. This pattern was of the same character as that of the other patient with hypopituitarism, case D.M., on ACTH administration. However, when one considers endogenous corticosteroid excretion values it is apparent that the response in the patient, case A.L., on ACTH administration alone was still somewhat greater than that observed for the patient, case D.M. Tetrahydro-F and tetrahydro-E were by far the major metabolites. The excretion of cortisone was somewhat greater than that of hydrocortisone while the amounts of the $C_{21}O_4$ corticosteroids excreted in the urine of the patient, case A.L., were approximately the same as when cortisone was administered alone.

As it was not possible to obtain the urinary corticosteroid pattern in the untreated disease state, case W.R., a patient with severe panhypopituitarism, was studied when on cortisone therapy. Thus the pattern obtained was essentially that of the metabolites of the administered cortisone which is discussed later.

The separate chromatography of the free and the enzyme hydrolysed fraction supplied some additional information. The finding of the greatest part of the reduced metabolites tetrahydro-F

and tetrahydro-E in the enzyme hydrolysed fraction was to be expected. It is of interest to note that with extensive extraction prior to hydrolysis apparently all of the hydrocortisone and most of the cortisone were recovered in the enzyme hydrolysed fraction suggesting that these compounds may have been excreted in some conjugated form. The finding is then similar to that of DeCourcy et al. (122) who were able to extract most of the cortisone from a pregnancy urine studied only after acid hydrolysis. Normally however, the biologically active compounds, cortisone and hydrocortisone, are recovered in greatest part from the free fraction.

The striking feature of the urinary corticosteroid pattern in the two cases studied with Cushing's syndrome due to bilateral adrenal hyperplasia was the great urinary excretion of the glucocorticoids compounds E and F when compared with the normal pattern. The finding of abnormally high amounts of the reduced metabolites, tetrahydro-F and tetrahydro-E, in the urine of patients with Cushing's syndrome would then be expected. Significantly, in neither of these patients studied in the untreated state was there any such increase above normal in the urinary excretion of the biologically active corticosteroids such as compounds B or A and of their reduced metabolites, the X_8 complex.

These results might indicate that the main defect in adrenal cortical activity in Cushing's syndrome is the abnormally high secretion of the glucocorticoids such as hydrocortisone and cortisone into the circulation. In fact, the actual rate of secretion of the biologically active corticosteroids has been measured in this disease by Bush (34). He has found the ratio of compound F to compound B to be 20 to 1 in the plasma of adrenal venous blood in a patient, which is considerably greater than that of 11 to 1 estimated by Pincus et al. (34) in normal adrenal venous blood. In addition, Dorfman (35) has attributed the characteristics of the clinical manifestations in this disease, such as diabetes and hypertension, to the high circulating levels of F-like compounds. Consequently, the diabetic symptoms observed in both of the patients with Cushing's disease, cases S.K. and G.A., and also the hypertension observed in the patient, case G.A., may be related to the finding, in this study, of increased urinary excretion of the $C_{21}O_5$ corticosteroids. These results are similar to those recorded in the literature by Wilson et al.(112) and Dohan et al.(34) who have also found that the most characteristic feature of the urinary corticosteroid pattern of Cushing's syndrome was the increase above normal in the excretion of the $C_{21}O_5$ corticosteroids and their metabolites.

The urinary corticosteroid patterns obtained in this study showed that both of the patients with Cushing's syndrome responded markedly to the administration of ACTH. This hyperactive response was to be expected in Cushing's syndrome due to bilateral adrenal hyperplasia according to Laidlaw (59) and, is in fact, regarded as a positive diagnosis for this disease. Eik-Nes (127) and Wallace (108) have observed a similar response in the blood. In both of the cases studied in this investigation there was a great increase in the urinary excretion of the biologically active corticosteroid hydrocortisone and, to a lesser extent, an increase in the urinary output of cortisone. In the patient, case S.K., the levels of tetrahydro-F and tetrahydro-E excreted were also increased. Consequently, there was an obvious similarity in both cases of Cushing's syndrome with respect to abnormally high excretion of the F and E-like compounds in the urine. However, in the patient, case G.A., but not at all to the same degree in the patient, case S.K., was there noted as well a marked response to ACTH administration with respect to increased urinary excretion of the $C_{21}O_4$ corticosteroids and their metabolites, the X_8 complex, which probably included in greatest part the components tetrahydro-S and tetrahydro-B. However, different ACTH preparations were used and thus as

according to Querido (82) it may be difficult to compare the absolute response in the two patients studied.

The finding of detectable amounts of the 17-hydroxylated corticosteroid, compound X_3, only in the pattern of the two patients with Cushing's syndrome on ACTH administration is of interest. This observation together with the apparent increase of a component of the X_8 complex which may have been tetrahydro-S in the pattern of the patient with Cushing's syndrome, case G.A. and also in the pattern of the patient with hypopituitarism, case A.L., on simultaneous cortisone and ACTH administration may be related to that observation made by DeFillipis (133) that on ACTH administration the increase in urinary corticosteroid excretion is mainly due to the greater output of compounds with the 17-hydroxy grouping, in addition to the primary alpha-ketol configuration. These results might indicate that ACTH directs adrenal cortical activity towards the secretion of the biologically active corticosteroids which possess the 17-hydroxy group.

The unusual feature in the urinary corticosteroid pattern of the patient with Cushing's syndrome, case G.A., after right adrenalectomy when receiving 200 mgm. of cortisone per day was the absence of the more polar $C_{21}O_4$ corticosteroid on the pattern. There was also a decreased excretion of tetrahydro-F, tetrahydro-E and cortisone below the levels observed in the un-

treated disease state despite the high cortisone dosage administered whereas the levels of hydrocortisone were approximately the same. It is interesting to speculate whether these results might indicate that the manifestations of Cushing's syndrome in this particular patient, case G.A., were due mainly to the hyperfunction of the right adrenal gland alone while the left adrenal was inherently incapable of secreting significant amounts of the corticosteroids. On the other hand, it is more likely that both adrenal glands had approximately the same degree of activity, but the function of the left gland was almost completely supressed by the high cortisone dosage administered.

The urinary corticosteroid pattern of the patient with Cushing's syndrome, case S.K., after total adrenalectomy represented only that of the metabolites of the administered cortisone, 50 mgm. per day. It illustrated that the greatest part of cortisone recovered was reduced in ring A to form the tetrahydro derivative compound THE. Relatively minor amounts of tetrahydro-F were excreted. This apparent difference in the quantitative pattern of these metabolites was observed in only one other type of pattern studied, that of the normal subjects. Small amounts of cortisone were converted into hydrocortisone by reduction of the ketone group at C_{11} and excreted as such while somewhat greater quantities were excreted unaltered. The urinary corticosteroid pattern also indicated clearly that (1) the

compounds X_1, X_2 and X_6 were metabolites of cortisone, (2) compound E was not degraded in metabolism to the biologically active $C_{21}O_4$ corticosteroids or their reduced metabolites to any significant extent. This has also been observed by other investigators including Burton et al.(16), (3) the characteristically occurring compounds X_9, X_{10} and X_{11} were not derived from cortisone. They were apparently of adrenal origin and were excreted in the urine, more or less at a steady rate, independent of the nature of the physiological state studied in this investigation.

It is of interest to point out that the metabolic pathways studied in this investigation would account for only a small fraction of the administered hormone. This was more evident by a study of the urinary corticosteroid pattern of the patient, with Cushing's syndrome, case G.A., after right adrenalectomy when receiving a high cortisone dosage of 200 mgm. per day. The bulk of the cortisone was apparently metabolized in other ways.

The urinary corticosteroid pattern of the patient, case F.T., in the untreated state of congenital adrenal hyperplasia complicated by episodes of severe hypertension was unusual in that the X_8 complex, probably including in greatest part the compound tetrahydro-S was by far the major metabolite while relatively minor amounts of tetrahydro-E and tetrahydro-F were

excreted. In contrast, the latter metabolites were predominant in normal urine and only small amounts of X_8 could be seen in the male pattern. In greater contrast in the cases of Cushing's disease there were far greater amounts of the $C_{21}O_5$ corticosteroids and their metabolites excreted in the urine than of the less polar compounds. Consequently, the paper chromatographic findings might be interpreted to indicate adrenal cortical dysfunction in the untreated disease state manifested by the secretion of subnormal amounts of the compounds F and E and abnormally high levels of one or more of the $C_{21}O_4$ corticosteroids into the circulation.

In some respects the urinary corticosteroid pattern of the case of virilizing hyperplasia studied in this investigation resembles that of the type of congenital adrenal hyperplasia, uncomplicated by hypertension, investigated by Eberlein et al.(36) (37), in that the urinary excretion of the metabolites compounds THE and THF was considerably decreased below normal. However, these authors could not find any significant amounts of the compound S or its reduced metabolite either in the blood or in the urine of the patients studied.

On the other hand, these investigators have studied a single case of congenital adrenal hyperplasia also complicated by hypertension in which the predominant C_{21} steroid was tetrahydro-S and had been estimated to be excreted at the rate of 18 mgm. per 24 hours. In this way the pattern was apparently similar

to that of the patient with congenital adrenal hyperplasia studied in this investigation. However, in variance with the urinary corticosteroid pattern observed in the patient, case F.T., no traces of compounds F, B or their metabolites were found, thus indicating an absolute lack of 11 hydroxylated steroids in the urine.

On cortisone therapy the unusual corticosteroid pattern of the patient with congenital adrenal hyperplasia studied in this investigation disappeared. The output of the complex, X_8, was almost completely supressed, the compounds THE and THF once more formed the bulk of the alpha-ketolic steroid material excreted and cortisone was clearly present in the pattern. The effect of cortisone therapy in returning the urinary corticosteroid pattern in virilizing hyperplasia to normal has been observed by a number of investigators including Wilkins et al (117) (124) and Golberg (46).

VI. SUMMARY AND CONCLUSIONS

The urinary corticosteroid pattern had been studied by the technique of paper chromatography in two normal subjects, three patients with hypopituitarism, two cases of Cushing's syndrome and in one case of congenital adrenal hyperplasia. The influence of cortisone and ACTH administration upon urinary corticosteroid excretion was investigated.

In one male and one female subject studied the results indicated that the major urinary metabolite was tetrahydro-E. Lesser amounts of tetrahydro-F were excreted while relatively small amounts of the biologically active corticosteroids compounds F and E were excreted unaltered. In both subjects the urinary excretion of the $C_{21}O_4$ corticosteroids and their reduced metabolites was considerably less than that of the more polar $C_{21}O_5$ steroids.

In one patient investigated in the untreated state of hypopituitarism there were excreted subnormal amounts of adrenal cortical hormones and their metabolites. However, the urinary corticosteroid pattern was of the same character as that of the normal subjects showing that tetrahydro-F and tetrahydro-E were the predominant constituents. Only small amounts of the gluco-corticoids compounds F and E were excreted and in proportion, there were far lesser quantities of $C_{21}O_4$ than of $C_{21}O_5$ cortico-

steroids excreted in the urine.

In two patients with Cushing's disease due to bilateral adrenal hyperplasia the outstanding feature of the urinary corticosteroid pattern was the presence of abnormally high amounts of the biologically active corticosteroids, cortisone and hydrocortisone and of their metabolites, tetrahydro-F and tetrahydro-E. There was no corresponding increase in the amount of $C_{21}O_4$ corticosteroids and their reduced metabolites, the X_8 complex, excreted in the urine.

In one patient with congenital adrenal hyperplasia complicated with episodes of hypertension the urinary corticosteroid pattern indicated the predominance of the X_8 complex, probably including in greatest part the metabolite tetrahydro-S. Only subnormal amounts of the $C_{21}O_5$ corticosteroids, compounds THE, THF and F were found.

The effects of ACTH administration varied in different patients with hypopituitarism. There was a striking change in the urinary corticosteroid pattern of one patient when ACTH was administered with cortisone. Abnormally high amounts of both $C_{21}O_5$ and $C_{21}O_4$ corticosteroids appeared in the urine. A much lesser response was observed in the same patient when a different

long-acting ACTH preparation was administered alone. A negligible response to ACTH was observed in another patient with hypopituitarism as evaluated by urinary corticosteroid excretion.

In two patients with Cushing's disease due to bilateral adrenal hyperplasia ACTH administration caused a hyperactive response leading to qualitative and quantitative changes in the urinary corticosteroid pattern. In one patient these changes in the pattern were mainly restricted to the compounds F,E and their metabolites. In another patient ACTH administration effected as well a marked increase in the urinary excretion of the less polar $C_{21}O_4$ corticosteroids which may have included such compounds as tetrahydro-S and tetrahydro-B.

The urinary corticosteroid pattern was studied on cortisone administration in two patients with hypopituitarism, two cases following total and right adrenalectomy and in one case of congenital adrenal hyperplasia. The results indicated that the major part of the cortisone recovered appeared in the reduced tetrahydro form; relatively small amounts of cortisone were converted to hydrocortisone and excreted as such. In cases with lowered adrenal cortical function there was a tendency for smaller amounts of cortisone than of hydrocortisone to be excreted unaltered,

but after total adrenalectomy more compound E then compound F appeared in the urine. The metabolites designated as X_1, X_2 and X_6 in this study were shown to be derived from E-like compounds while the less polar compounds X_9, X_{10} and X_{11} were shown to be of adrenal origin. Cortisone was not degraded in metabolism to the biologically active $C_{21}O_4$ corticosteroids and their reduced alpha-ketolic metabolites.

In addition to showing the metabolites of the compound E the influence of cortisone therapy upon the urinary corticosteroid pattern was studied in one case of congenital hyperplasia. On cortisone administration there was almost complete supression of the abnormally high excretion of the X_8 complex observed in the untreated disease state and the urinary corticosteroid pattern became normal in appearance.

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