A FLUORIMETRIC DETERMINATION
OF UNCONJUGATED CORTISOL IN HUMAN URINE
AFTER PAPER CHROMATOGRAPHY

By
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ABSTRACT
A method for the estimation of unconjugated cortisol concentration in human urine is described. Using highly sensitive fluorimetric assay after paper chromatographic purification of cortisol, it is possible to quantitate the amount of endogenous cortisol excreted in 24 hours urine, after steroid suppression and corticotrophin stimulation (ACTH). According to this method, normal values (30 males and 30 females aged between 18–50 years) are 35.8–120 µg/24 hours; fluorimetric recoveries of cortisol from urine are 82.5–95.4% and radioactive recoveries are 74.8–88.6%. A trained person can perform 12 determinations in 5 working days.

In recent years fluorimetric methods have been published for the determination of cortisol and corticosterone in plasma, to assess the adrenal cortical function under various pathological and experimental conditions (De Moor et al. 1960; De Moor & Steeno 1963; Braunsberg & James 1960 a, b, 1962; Stewart et al. 1961; Mattingly 1962; Rudd et al. 1963; Daly & Spencer-Peet 1964; Cameron & Kilburn 1964; Vermeulen & Van der Straeten 1964; Spencer-Peet et al. 1965). Not much work has been published regarding the determination of unconjugated cortisol in urine, those in common use require large volumes of urine to be extracted and finally estimated colorimetrically (Crabbé et al. 1958; Cope & Black 1959; Greaves & West 1960; Ross 1960; Franken & Zimmernann 1962; Cost & Vegter 1962; Rosner et al. 1963; Brooks et al. 1963). It has been concluded that the simplified fluorimetric methods (Gantt et al. 1964; Mattingly et al. 1964) offered adequate sensitivity, precision and accuracy, but their specificity is open to question as crude plasma or urine extract
contains considerable amounts of fluorescent substances other than cortisol and corticosterone. Fluorimetric methods of determining cortisol are ten times more sensitive than the Porter-Silber reaction (Nelson & Samuels 1952). So, there remained a need for development of a valid specific assay of urinary unconjugated cortisol. Methods applying fluorimetric determination after chromatographic purification are more specific and could be of value in some biological and clinical problems.

Paper chromatography has been extensively used for the separation and purification of steroids (Peterson 1957; Bondy et al. 1957 a; Bondy & Upton 1957 b) using Bush (1952) system; (Burton et al. 1951; Abelson & Brooks 1960). The application of paper chromatography has two disadvantages to determine the small quantity of steroids present. (a) The value of the blank becomes important and this has to be reduced. The type of treatment required is related to the method applied for the determination of the steroid. It is necessary to make sure that the blank is sufficiently low and uniform over the length of the paper. (b) The problems of determination of steroid in a specific situation after chromatography or of satisfactory quantitative elution from paper before assay have not been solved (Abelson & Brooks 1960). Paper chromatography offers great advantage when large numbers of samples are to be analysed. This study provides a specific method to determine the unconjugated cortisol in urine.

**REAGENTS**

Ethyl acetate, petroleum spirit b. p. 60–80° C, benzene, methylene chloride, toluene (Hopkin and Williams Ltd.) GPR grade were further purified according to the methods described by Vogel (1957).

Sodium chloride, silver nitrate, potassium hydroxide pellets, sulphuric acid, S. G. 1.84, sodium hydroxide pellets were of ANALAR grade (Hopkin and Williams Ltd.).

Potassium dichromate, sodium metabisulphite were of laboratory reagent grade (British Drug Houses Ltd.).

Ethanol (James Burrows Ltd.), absolute alcohol R. R. grade was purified in the following way:

In a 3 litre round bottom flask 1 g of potassium hydroxide pellets and 10 g of silver nitrate per litre of ethanol were refluxed for 6 hours and then distilled twice through a Dufton column, fractions were collected at 78° C and stored in dark bottles at 4° C.

Glass distilled water was used throughout the work.

Cortisol was further purified by chromatography in benzene 1000: methanol 500: water 500 system of Bush (1952).

Cortisol stock standard 2 mg of cortisol (Medical Research Council reference compound) was weighed on a microbalance and dissolved in purified ethanol and volume was made up to 100 ml. This was stored at 4° C. 1 ml (20 µg) of this solution was diluted with distilled water and volume was made up to 200 ml (1 ml = 0.1 µg). This was the working standard solution and was made fresh before use.
Cortisol acetate. (Medical Research Council reference compound).

Glass stoppered test tubes 15 × 2.5 cm, approximate capacity 54 ml, were used. (Quickfit & Quartz Ltd.).

A horizontal mechanical shaker was used for shaking. A «Griffin» filler was used all through for pipetting. Centrifuging was done at 3000 r. p. m.

It is essential to keep all glassware absolutely clean.

After standing in chromic acid overnight and washing with cold tap water followed by an aqueous solution of 5 % sodium metabisulphate and cold tap water, the glassware was finally washed with distilled water and segregated for this work only.

75 % (v/v) ethanol-sulphuric acid reagent was prepared by slowly adding 3 vol. ice-cold conc. H₂SO₄ to 1 vol. ice-cold purified ethanol, the mixture being cooled in ice water.

Fluorescence measurements were performed in an Aminco-Bowman Spectrophotofluorimeter (American Instrument Company Inc., Silver Spring, Maryland, U. S. A.) at zero sensitivity and meter multiplier set at 0.003, using silica cuvettes of 1 cm light path (external dimensions: 1.2 cm square × 4.8 cm high, internal volume 4.8 ml) at fluorescent wavelength 530 mµ, activating wavelength 470 mµ. No filters were incorporated in the light path. The slit system was as follows: No. 1, 1/8 in.; No. 2, 1/16 in.; No. 3, 1/8 in.; No. 4, 1/8 in.; No. 5, 1/16 in.; No. 6, 1/8 in.; photomultiplier slit, 1/16 in. A 1P 21 photomultiplier tube was used. In certain experiments a Moseley Autograf Model 1 X-Y recorder (F. O. Moseley Co., Pasadena, California, U. S. A.) was coupled to the fluorimeter.

Radioactive measurements were carried out in a Packard TriCarb Liquid Scintillator Spectrometer, Model 314, EX. (Packard Instrument Company Inc., P. O. Box 428, La Grange, Illinois, U. S. A.). The counting vials were supplied by Packard Instrument Company.

The scintillator was a mixture of solution of 0.4 % diphenyloxazole (PPO) and 0.05 % Dimethyl POPOP-1,4-bis-2 (4-Methyl-5-Phenylxozoly)-Benzene, scintillator grade (Packard Instrument Company) in purified toluene.

Stock cortisol-4-¹⁴C (The Radiochemical Centre, Amersham, Buckinghamshire, England). Specific activity 22.3 mCi/mM, 61.7 µc/mg, was diluted in purified benzene to a final concentration of 0.1 µc (1.64 µg) of cortisol-¹⁴C per 1 ml of benzene and was stored in −10 °C.

**METHOD**

Urine was collected for 24 hour periods without any preservative and the pH was adjusted to 7.0, part of it was used for the determination of the total 17-ketosteroids and total 17-hydroxycorticosteroids according to Callow et al. (1938) and Few (1961). In both cases Zimmermann colorimetry was performed according to James & De Jong (1961). A 20 ml aliquot was pipetted out and sodium chloride added to give a final concentration of 20 % w/v and the urine extracted 3 times with equal volumes of ethyl acetate. The combined ethyl acetate layers were washed in turn with 0.05 vol. of 0.1 N sodium

417

Acta endocr. 51, 3
hydroxide solution, 0.05 vol. of 0.1 N glacial acetic acid and 0.05 vol. of water. The extract was then poured over anhydrous sodium sulphate supported on glass wool in a funnel and was evaporated to dryness on a rotary film evaporator in a 100 ml round bottom flask, in the bottom of which a small well had been blown, the sides of the flask were washed down with small quantity of methanol:ethyl acetate (1:1) into the well and the solvent finally removed by a stream of nitrogen at 50°C and the residue was dissolved into 0.02 ml of methanol and spotted along the origin of a 2.5 cm wide Whatman no. 2 paper. 20 ml of distilled water was extracted as blank, 0.2 µg, 0.4 µg, 0.6 µg, of pure cortisol in 20 ml of distilled water were taken as standards and were processed all through. A 5 µg amount of pure cortisol was spotted as a guide strip.

Chromatograms were developed in a Bush (benzene 1000: water 500: methanol 500 system, equilibrated for 2 hours, the solvent being run for 14 hours and the papers dried at room temperature (Bush 1952). The guide strip was dipped into alkaline blue tetrazolium reagent to detect the cortisol spot according to Pal (1964). Cortisol parts from the chromatograms were cut out and eluted with 10 ml of methanol at room temperature, eluates were transferred to glass stoppered tubes and were evaporated to dryness in a rotary film evaporator and the residue was dissolved into 20 ml of distilled water and 20 ml of petroleum ether was added from an automatic measure. The tubes were shaken for 5 minutes and then centrifuged for 5 minutes. The petroleum ether was separated and removed by suction and discarded. The aqueous layer was then extracted with 20 ml of methylene chloride, added from an automatic measure. The tubes were thoroughly shaken for 10 minutes and then centrifuged for 5 minutes. The aqueous layer was carefully discarded by suction.

15 ml of the methylene chloride extract was pipetted into another tube and was evaporated to dryness on a water bath at 40°C under nitrogen. The residue was dissolved into 15 ml of distilled water and 5 ml of benzene was added from a burette and the tube was again thoroughly shaken for 10 minutes. After centrifuging for 5 minutes the benzene layer was very carefully removed by suction with a capillary pipette. The water phase was extracted with 30 ml of methylene chloride and was shaken for 10 minutes and after centrifuging for 5 minutes the aqueous layer was discarded by suction. 1.5 ml of 0.1 N NaOH was added and the tube shaken for 1 minute. After centrifuging for 5 minutes the sodium hydroxide layer was removed by suction. 10 ml of the methylene chloride extract was pipetted out into another tube and was now ready for treatment with the ethanol-sulphuric acid reagent. All measurements were performed in duplicate and were repeated if the difference between the duplicate determinations was higher than 10%.

Fluorimetry was carried out by adding 5 ml of ethanol-sulphuric acid rea-
gent from a burette into 10 ml of methylene chloride extract and carefully mixing after releasing the pressure, by gentle inversion for 30 seconds. After allowing the two phases to separate, the methylene chloride layer was then removed by aspiration, and the ethanol-sulphuric acid layer was poured into the cuvette. Fluorescence was read at 10 minutes after mixing.

**Calculation**

The fluorescence of the extracts was examined in terms of fluorescence intensity (F.I.) given by multiplying the galvanometer reading by the meter-multiplier setting (Fig. 1). Urinary cortisol excretion per 24 hours is worked out as follows:

\[
\frac{\text{F.I. of test} \times \text{cortisol in standard} \times \text{vol. of 24 hour urine}}{\text{F.I. of standard} \times 10 \text{ or } 20 \text{ ml urine}}
\]

**Wave length**

The activation and fluorescence wave lengths were checked for solutions of pure cortisol reference compounds. Maximum fluorescence was obtained at 530 m\(\mu\) when the activation wave length was 470 m\(\mu\) at 10 minutes (Fig. 2). The activation and fluorescence spectra of normal urinary extracts, pathological urine at high and low concentrations were parallel (Figs. 3, 4 and 5) as observed with pure cortisol and extracts from cortisol added to urine.

![Fig. 1](image)

Standard cortisol calibration curve (in duplicate) taken all through the method.
Fig. 2.
Reference cortisol. 0.40 µg.

 Activation spectrum at Fluorescence wavelength 530 mµ.
 Fluorescence spectrum at Activation wavelength 470 mµ.

Fig. 3.
Normal urine.

 Activation spectrum at Fluorescence wavelength 530 mµ.
 Fluorescence spectrum at Activation wavelength 470 mµ.
Fig. 4.

Urine with a high concentration of cortisol (on ACTH).

\[ \text{Activation spectrum at Fluorescence wavelength 530 } \mu \text{m.} \]
\[ \text{Fluorescence spectrum at Activation wavelength 470 } \mu \text{m.} \]

Fig. 5.

Urine with a low concentration of cortisol.

\[ \text{Activation spectrum at Fluorescence wavelength 530 } \mu \text{m.} \]
\[ \text{Fluorescence spectrum at Activation wavelength 470 } \mu \text{m.} \]
Radioactive measurement

Active extracts were evaporated under nitrogen in a waterbath at 40° C directly in the counting vials. All counting vials containing samples were kept in the dark with scintillator solution for at least 3 days before counting. Sample measurements were corrected for quenching by adding an internal standard to each sample and again estimating the 14C with an average efficiency of 70 %. Background and active samples were counted to bring to 10 000 counts.

Collection, storage and stability of urine at room temperature (20° C)

Many samples of 24 hours' urine for analysis were collected by outpatients at home, without any preservative, mixed, the volume measured and part (120 ml) of the collection sent by post in 9 × 6 × 2 cm, size flat polythene bottles with screw caps. No urine sample was more than 48 hours old. To perform this task, patients were instructed by the laboratory staff, when they came to the clinic, and they were also provided with 2 litre capacity polythene measuring cylinders.

To find out the stability of cortisol in urine due to standing, a 20 ml aliquot of a collection of pooled urine standing at room temperature without preservative at 20° C was extracted every day for 14 days and cortisol was measured. There was no significant difference in results (Table 1).

<table>
<thead>
<tr>
<th>Day of assay</th>
<th>Cortisol in µg/24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>110</td>
</tr>
<tr>
<td>2</td>
<td>113</td>
</tr>
<tr>
<td>3</td>
<td>115</td>
</tr>
<tr>
<td>4</td>
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<td>7</td>
<td>116</td>
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<td>8</td>
<td>114</td>
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<tr>
<td>9</td>
<td>115</td>
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<td>10</td>
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<td>13</td>
<td>117</td>
</tr>
<tr>
<td>14</td>
<td>116</td>
</tr>
</tbody>
</table>
RELIABILITY OF THE METHOD

The reliability of the method was judged by the following criteria (Borth 1957).

Specificity
The specificity for cortisol was studied in terms of the specific activity of cortisol and for this $^{14}$C-labeled cortisol of known specific activity was used. A collection of pooled normal urine (volume 2 litres) was taken, cortisol concentration was determined, 400 ml aliquot of urine was taken, 0.05 µc (11 000 cpm)cortisol-$^{14}$C was added to it. Corticosteroids were extracted, chromatographed, eluted according to the method described. Dry residue of cortisol was acetylated (Bush & Willoughby 1957) and chromatographed in petroleum ether (100–120° C) 667: benzene 333: methanol 800: water 200 system of Bush (1952) for 16 hours at room temperature, eluted. The cortisol acetate residue was divided into three equal parts, the first part was counted for the activity, the second part was estimated for cortisol fluorimetrically against standard cortisol acetate solution, the third part was transferred into a glass stoppered tube and dissolved in 1.6 ml of methanol, nitrogen was passed through the methanol for 5 minutes. Cortisol acetate in methanol was hydrolysed with 0.4 ml of aqueous 2% potassium bicarbonate solution and the tube was stoppered, left in darkness overnight. Nitrogen was passed through the solution, one drop of 50% acetic acid and 3 ml of water were added, extracted three times with 5 ml of ethyl acetate, dried over 0.5 g of anhydrous sodium sulphate and evaporated to dryness on a water bath at 40° C under nitrogen. Fluorimetric determination was performed.

Specific activity = \( \frac{\text{counts per minute of cortisol acetate}}{\mu g \text{ of cortisol acetate fluorimetrically}} \) = 54 µc/mg

Similarly,

Specific activity = \( \frac{\mu g \text{ of hydrolysed cortisol acetate (cortisol) fluorimetrically}}{\text{counts per minute of cortisol acetate fluorimetrically}} \) 56.5 µc/mg

After performing several experiments (N = 12) specific activity of cortisol estimated agreed closely with that supplied by Radiochemical Centre and the results confirmed the high specificity of the described method for cortisol.

Accuracy
The accuracy of the method was assessed by determination of the recovery of cortisol added to normal urine and to distilled water. An aqueous solution of cortisol (0.1 µg/ml) was added to 20 ml aliquot of normal urine and to 20 ml of distilled water, to give additions of 0.2–0.6 µg. Under these conditions the mean recovery of cortisol from urine was 88.4% (S. D. = 8.6), N = 30, from distilled water was 90.2% (S. D. = 8.4), N = 30. Similarly
adding 0.005 µc of cortisol-¹⁴C, mean recovery from urine was 76.4 % (S. D. = 9.4), N = 30, from distilled water 77.8 % (S. D. = 8.2), N = 30. Percentage recovery of cortisol in urines were also determined at low-concentration as accuracy varied with concentration (Table 2).

**Precision**

A 24 hour normal urine was divided into 30 equal parts and estimation was performed on each sample. An estimate (s) of the standard deviation (σ) of the results (x₁, x₂, x₃ . . . . . . .) from their mean (x̄) may be obtained from

\[ s = \sqrt{\frac{\sum(x_\text{-}x)²}{n-1}} \]

where n is the number of replicate determinations.

S. D. = ± 4.3 from a mean value of 40.5 µg/24 hours.

As before, a 24 hour urine from a patient with rheumatoid arthritis on 10 mg of prednisolone per day was analysed (30 estimations):

S. D. = ± 5.1 from a mean value of 12.4 µg/24 hours.

**Normal values**

The unconjugated cortisol in the urine of 60 normal subjects (30 males and 30 females), from all walks of life and without any endocrine disorders, aged between 18–50 years, varied from 35.8–120 µg/24 hours. The mean value was 82.6 µg (male 82.4 µg, female 83 µg/24 hours) without taking body weight into account. The total 17-hydroxycorticosteroid level was 7.3–14.4 mg/24 hours and the total 17-ketosteroid level was 6.2–15.8 mg/24 hours, without taking body weight into consideration.

**Table 2.**

Recovery of added cortisol from urines with low concentration of cortisol.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Cortisol µg/24 h</th>
<th>Recovery of cortisol µg/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>W. F.</td>
<td>6.2</td>
<td>70.1</td>
</tr>
<tr>
<td>C. B.</td>
<td>9.3</td>
<td>71.2</td>
</tr>
<tr>
<td>L. F. H.</td>
<td>10.6</td>
<td>70.8</td>
</tr>
<tr>
<td>W. A.</td>
<td>11.1</td>
<td>71.2</td>
</tr>
<tr>
<td>N. R.</td>
<td>12.8</td>
<td>71.3</td>
</tr>
<tr>
<td>C. W.</td>
<td>13.3</td>
<td>72.1</td>
</tr>
<tr>
<td>F. B.</td>
<td>14.5</td>
<td>72.4</td>
</tr>
<tr>
<td>J. B.</td>
<td>15.7</td>
<td>72.7</td>
</tr>
<tr>
<td>H. W.</td>
<td>17.6</td>
<td>74.6</td>
</tr>
<tr>
<td>M. P.</td>
<td>19.2</td>
<td>76.8</td>
</tr>
<tr>
<td>D. F. L.</td>
<td>22.6</td>
<td>80.2</td>
</tr>
</tbody>
</table>
DISCUSSION

The aim of the present investigation is to establish a fluorimetric method of high specificity for the determination of endogenous cortisol in urine. As cortisol is a very polar compound, in order to avoid any incomplete extraction, a very polar solvent like ethyl acetate is used. The use of ethyl acetate gives better recovery of cortisol and is supposed to yield purer extracts (Meyer 1953; Robertson & Mixner 1956) though this has been disputed (Tamm et al. 1958). In a previous paper, Pal & Smith (1965) found that the use of methylene chloride was satisfactory for the extraction of cortisol in urine. The extraction was incomplete, however, and the recovery was less from the urines of pregnant women, Cushing’s syndrome, adenoma, adrenal hyperplasia, and from the urines of patients on ACTH, butazolidin, aspirin, sulphadimidine, hyperlipaemia and hypercholesterolaemia; hence methylene chloride was replaced by ethyl acetate in this procedure.

Fluorimetry is an elementary analytical technique for the assay of the fluorescent materials. It is extremely sensitive and relies on the proportionality between the concentration and the fluorescent intensity of a dilute solution, excited by radiation of constant intensity and spectrum, and observed through an appropriate filter. Solvent effects, impurities, temperature, pH and photochemical effects are the factors which affect the fluorescence intensity and spectrum. The fluorimetric method appears to afford a reliable measurement of cortisol concentration in urine and blood which shows several desirable characteristics. It is readily and quickly performed and requires a minimum technical manipulation and is capable of an acceptable degree of precision and accuracy. The relatively great sensitivity of the method makes it uniquely applicable in studies where a very small quantity of materials is available.

The present study has been primarily designed to investigate and quantitate the excretion of urinary endogenous cortisol and is applied to patients with rheumatic disorders on prolonged ACTH and oral synthetic corticosteroid therapy.

As fluorimetric methods are very sensitive, small changes in experimental conditions and impurities present could make the results misleading. Impurities may combine with the fluorescent substance and form compounds which fluoresce more or less like the compound to be estimated. In such a case it is difficult to use a fluorimetric method to its maximum advantage without introducing any procedure of purification of the extract.

Applying non-specific nature of isolation procedure of cortisol and careful timing (Mattingly 1962; Mattingly et al. 1964; Gannt et al. 1964), mathematical extrapolation (Daly & Spencer-Peet 1964; Spencer-Peet et al. 1965) of the sulphuric acid-ethanol induced fluorescence of the extract without any purification does not solve limitations of specificity of cortisol. So a reliable and
relatively rapid purification procedure has been introduced involving paper chromatography, solvent partition and mild alkaline wash to remove the non-specific materials, hence making the fluorescence of cortisol more specific.

Fluorescence produced by extracts presents some difficulties when fluorimetry is performed after paper chromatography. The value of the paper blank is very important and should be kept as low and uniform as possible throughout the chromatogram. Application of this method gave the same fluorescent intensity as the reagent blank and is uniform from 6 different portions from the same chromatogram (N = 12).

Oestrogens produce strong fluorescence in sulphuric acid (Bates & Cohen 1947; Jailer 1947); and many other steroidal compounds fluoresce under similar conditions (Bandow 1938 a, b, 1939). 11β-Hydroxyandrostenedione also produces fluorescence of intensity comparable to that given by cortisol (Braunsberg & James 1960 a, b). The method adopted for purification of the extract is essential because of the presence of some amounts of solid material in the cortisol fraction obtained from the urine of the rheumatic patients who are usually on a large dose of aspirin or butazolidin per day. These residues quenched the fluorescence of cortisol approximately 10%, hence making the analysis less accurate.

Patients suffering from rheumatoid arthritis, treated with ACTH, excrete appreciable amounts of 6β-OH cortisol (a metabolite of cortisol which passes through the liver without being conjugated with glucuronic acid or sulphuric acid) which is excreted in urine as a free state which also fluoresces under the condition, and a higher reading is consistently observed. Introduction of paper chromatographic separation was obviously necessary to remove that compound.

The relative specificity of the fluorescence reaction which has been described by several workers (Sweat 1954; Zenker & Bernstein 1958; De Moor et al. 1960; De Moor & Steeno 1963; Braunsberg & James 1960 a, b; Stewart et al. 1961; Mattingly et al. 1964), may only relate to a limited number of possible variations in time, temperature and sulphuric acid-ethanol concentration. It is absolutely necessary that the condition of the reaction be strictly controlled using the condition similar to Sweat (1954.) The above-mentioned workers have confirmed that the production of significant fluorescence is limited to a relatively small number of steroid configurations.

In the urine of 419 normal subjects (De Moor & Steeno 1963) reported a mean value of unconjugated cortisol of 191 µg/24 hours (female 179 µg, male 210 µg/24 hours). The mean urinary free 11-hydroxycorticosteroid excretion of 86.8 ± 29.5 (S.D.) µg/24 hours in 17 healthy adult subjects between the ages of 20 and 40 years with a range of 31 to 150 µg/24 hours is reported by Gantt et al. (1964). In the urine of 24 normal subjects Pal & Smith (1965) reported a mean value of 93 µg/24 hours (female 89.5 µg, male 97.2 µg/24
hours). These results are without taking body weight into consideration. The values reported by the above authors in the urine of normal subjects are higher than the values stated in this communication as they did not involve the application of paper chromatography and other purification procedure of the cortisol extract.

Although the method presented here for the estimation of cortisol requires careful purification, it has useful application in assessing the adrenal cortical function in a given individual under ACTH stimulation and acute corticosteroid suppression with a high degree of specificity and accuracy.

ACKNOWLEDGEMENTS

I wish to thank Dr. W. S. C. Copeman and Dr. Oswald Savage, Consultant Physicians to the Institute, for the opportunity to study their patients; Professor W. Klyne, Westfield College, for generously providing reference compounds from the Medical Research Council reference section; Dr. E. S. Garnett, Fulham Hospital, for counting the radioactive samples; Professor J. Patterson, Charing Cross Hospital Medical School, for his valuable suggestions; Mr. R. Gunascgaram and Mrs. M. P. Rake for performing some steroid estimations; Miss E. Gask, Research Secretary, and Mrs. M. R. Pal, for help in preparing the manuscript.

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Received on June 14th, 1965.