Numerous steroids have been isolated from human urine in recent years and their structure is now to a great extent established. Some of them are derived from the gonads but most of them are probably metabolites of the steroid hormones elaborated by the adrenal cortex. These include the biologically active corticoids (glucocorticoids with a specific effect on protein and carbohydrate metabolism, and mineralocorticoids with a specific effect on water and electrolyte metabolism), androgens, oestrogens and progesterone. In addition to these, however, there are a large number of biologically inactive steroids closely related to the above mentioned groups.

Up to quite recently the chemical nature of the biologically active substances in the urine, especially the so-called glucocorticoids, was unknown. In 1948 Mason et al., however, isolated large amounts of 17-hydroxycorticosterone in the urine from a patient suffering from Cushing’s syndrome and recently Schneider (1950) isolated 55.3 mg. 17-hydroxy-11-dehydrocorticosterone (cortisone) from 1000 litres of normal male urine. The excretion per 24 hours was calculated to be of the order of 82 µg.

Various biological as well as chemical methods have been proposed for the quantitative determination of the urinary corticoids (reviewed by Sprechler, 1949). In practice the most
useful biological tests are those based on the capacity of the
gluocorticoids to cause glycogen deposition in the liver of the
fasting adrenalectomized animal. In this respect the method
of Venning et al. (1946) appears to be one of the most sen-
sitive.

Two types of chemical methods have been worked out. One
of these is based on the reducing property of the α-ketol side
chain at C₁₇ for the cupric ion (Talbot et al., 1945) and for
phosphomolybic acid (Heard & Sobel, 1946). The last-men-
tioned reagent also reacts with the α, β-unsaturated keto
group at C₃. These two groupings are always present in the molecule
of the typical biologically active corticoids. The other type of
method (Lowenstein et al., 1946, Daughaday et al., 1948) de-
ponds on the measurement of the formaldehyde released on
periodic oxidation of the primary α-ketol grouping at C₁₇. In
addition, however, formaldehyde can also be produced from
steroids hydroxylated at C₂₁.

OWN INVESTIGATIONS

The aim of the present study was to re-examine the existing
chemical methods for the determination of urinary corticoids.

At first the most simple method devised by Heard, Sobel &
Venning (1946) was explored. More than 500 analyses were
carried out in the course of one year. Several of these were per-
formed in duplicate, and after some practice a good agreement
was obtained between the two assays. In several cases, however,
different coloured substances passed from the urine into the
lipoid solvent and interfered with the blue colour in the colori-
metric assay. It has not been possible to obtain a correction
for this unspecific colour. Furthermore very large day to day
variations were found in the results of analyses carried out
on hospital specimens from patients kept under uniform con-
ditions in medical departments. This must have been due to
unspecific reducing substances and was not found when using
a method which will be described later. Heard, Sobel & Ven-
ning (1946) suggested in an addendum to their paper that
special precautions must be taken during the collection of urine in order to avoid contamination. This is impracticable if a large number of analyses have to be performed by routine procedure and as it was not possible to obtain reliable estimates, especially for routine purposes the method was abandoned.

Subsequently the method of Talbot et al. (1945) was investigated. In this troublesome and time-consuming method a thorough fractionation of the crude urinary extract is necessary before the final colorimetric assay. In all the experiments, however, insuperable difficulties were encountered in the final chemical procedure, because of precipitation in and cloudiness of the reaction mixture. Some investigators have suggested that these insoluble substances should be extracted by shaking with ether, but this was found quite unsuitable as it gave unreliable results in control assays. Heard & Sobel (1946) observed the same difficulties and ascribed them to extreme insolubility of most steroids in the aqueous alkaline copper solution. The same investigators also stated that a benzene-water partition is not specific for the glucocorticoids as suggested by Talbot et al., who found that 17-hydroxycorticosterone and 17-hydroxy-11-dehydrocorticosterone were recovered quantitatively from the aqueous phase, while corticosterone and 11-dehydrocorticosterone remain mostly in the benzene (57 and 71 per cent respectively). Heard and Sobel also stated that an 11-desoxy-compound, $\Delta^4$-pregnene-17, 20, 21-triol-3-one, is recovered in water.

The method based on the formaldehyde liberating power on the periodic oxidation could not be examined here as we were unable to obtain the necessary reagents. The results mentioned above led us to base the chemical assay on the principles described by Heard & Sobel but in order to get a higher specificity in the determination, we have performed a further fractionation of the total neutral urinary extract as proposed by Talbot et al. Girard's reagent T has been used for this purpose. The various steps in the procedure have been critically studied, and the results of these experiments are given below.
REAGENTS

Sodium hydroxide, sulphuric acid, glacial acetic acid and anhydrous sodium sulphate are all analytical reagents. Chloroform (Ph. Dan.) is freshly redistilled.

Girard's reagent T (Ciba) must be kept in a dessicator or in ampoules.

Phosphomolybdic acid solution (Folin & Wu, 1920): 35 gm. molybdic acid and 5 gm. sodium tungstate are dissolved in 200 ml. 10 per cent sodium hydroxide and 200 ml. of distilled water are added. In order to remove the ammonia it is boiled vigorously for 20—40 minutes. After cooling and dilution to 350 ml. with distilled water 125 ml. of concentrated phosphoric acid are added. Finally the solution is diluted to 500 ml. with distilled water.

Phosphomolybdic acid reagent (Heard & Sobel, 1946): Immediately before use, the reagent is made by mixing equal parts of the above-mentioned phosphomolybdic acid solution and glacial acetic acid.

Molybdic acid is prepared as follows: A thin layer of ammonium molybdate is placed in a pyrex glass tube of high melting point, through which a stream of oxygen is passed. On intensive heating the ammonium molybdate is transformed into needles and into leaves of molybdic trioxide ($\text{Mo}_3\text{O}_9$). By the use of this technique it has been possible to obtain a reagent that is stable for a long time when kept in the cold and in the dark.

Several reducing substances react with the phosphomolybdic acid and it is, therefore, essential to take every precaution when performing the analyses. The lubricants used must be controlled. The glass-ware must be cleaned by washing with water, alcohol and ether. All distillations must be carried out in all-glass apparatus. Last but not least, the inside of the glass-ware and the tips of funnels etc. should not be touched. In order to control the specificity of the analysis itself, blank determinations have been performed.
THE URINE

A 24-hour urine specimen is collected without preservative and kept in the cold up to the period of extraction, which should be performed as soon as possible. If this cannot be done, the urine should be kept frozen in a refrigerator.

If a normal content of corticoids is expected, $\frac{1}{10}$ of the 24-hour specimen is used for the analysis. If this amount is less than 80 ml, the urine is diluted to this volume with distilled water, as it is an advantage to work with not too small an amount of urine.

ACIDIFICATION

Talbot et al. (1945) extracted unacidified urine but Heard, Sobel & Venning (1946) and others obtained considerably higher yields from acidified urine assayed chemically. This has been confirmed by Venning et al. (1946) who used a biological test for assaying the glucocorticoids. These investigations show that some of the urinary corticoids must be present in a conjugated form.

In order to find the optimal pH value several experiments at different pH levels have been performed on various urines, the extraction being performed immediately after acidification. In some instances the total neutral extract has been divided into two equal parts. One of these is treated with Girard's reagent as described later, the other is partitioned between benzene and water, using the method of Talbot et al. (1945), after which the reducing power of the total water-soluble steroids is determined.

In both cases the yield was 75—100 per cent higher at pH 1 as compared with the result obtained by the extraction of unacidified urine (Fig. 1). The maximum liberation of corticoids from their conjugates seems to take place at a pH of between 2 and 1.
The effect of acidification of the urine on the extraction of corticoids. The columns represent the average result of experiments carried out on 5 different urines.

Aliquots of 24-hour from 6 women and 5 men were also adjusted to pH 1 and pH 2. From the results obtained by extraction at pH 1 and pH 2, respectively, the ratio of pH 1 : pH 2 has been calculated (Table 1). On an average these ratios appear to correspond closely with the results illustrated in Fig. 1. It is of interest that the ratio is found to be higher for men than for women. Heard, Sobel & Venning (1946) observed a similar sex difference with regard to the absolute excretion values.

It must be assumed that the acidification causes a mild hydrolysis. It is, therefore, of interest to determine the effect of time on this hydrolysis. Several analyses were performed in the following manner: Large amounts of urine were adjusted
The urinary excretion of corticoids in 6 women and 5 men. The extraction was carried out at pH 1 and pH 2.

to pH 1 and then divided into a number of equal portions. Extraction was carried out immediately after the acidification and then hourly for the following 8 hours, and finally after standing for 24 hours at room temperature.

Such large variations from one urine to another were found that it was impossible to give any general rules for the optimum time of hydrolysis. After 5—6 hours, however, increasing amounts of extractable reducing ketonic corticoids were found in most cases. The highest values appear to be reached after 24 hours' hydrolysis, but biological determinations performed simultaneously showed lower values for the content of glucocorticoids, indicating a destruction or a conversion of the biologically active corticoids into inactive products.

In order to eliminate this destructive effect of the acid upon the labile part of the corticoids, it was decided to carry out the extraction immediately after the acidification to pH 1 (measured with a glass electrode) and using 40 vol. per cent H₂SO₄, though we realised that these were not the optimal conditions.
EXTRACTION

Heard, Sobel & Venning (1946) used a chloroform-ether mixture 1:4. Talbot et al. (1945), however, found ether less suitable as the partition between this solvent and water for several steroids is 1:1. They found that a small volume of chloroform extracted practically all the corticoids.

Experiments have shown that even the most purified ether available to us (Ph. Dan.) contains some unspecific reducing substances, which are not completely removed by redistillation. Redistilled chloroform, however, gives satisfactory results, as shown by several control analyses (»water blanks«). It is of interest that, when working with Heard & Sobel's method, we always found much higher values for the urinary content of corticoids when chloroform was used as the extraction solvent.

Several assays (Table 2), performed with different volumes of chloroform, show that satisfactory recoveries can be obtained by shaking the urine vigorously three times with 25 per cent (by volume) of chloroform in a separating funnel. The emulsions, which are practically always formed, are broken down by centrifugation. If the urine contains large quantities of pus and albumin it is necessary to centrifuge it before the extraction. The chloroform extracts are combined in the separating funnel and washed by shaking three times with 10 ml. of 0.1 N NaOH and then three times with 10 ml. of distilled water. Each washing is extracted back with 5 ml. of chloroform and these are added to the original chloroform extract before going on with the next washing. This re-extraction is especially necessary as the NaOH will otherwise cause a small loss each time. Several yellow-brown coloured substances and phenols are removed by shaking with NaOH. These substances can interfere unspecifically with the colorimetric assay.

The chloroform extract is dried with small amounts of anhydrous sodium sulphate, filtered and evaporated to dryness in an all-glass vacuum distillation apparatus, the temperature of the water-bath being kept at 45° C.
Table 2.

<table>
<thead>
<tr>
<th>Chloroform per cent (by volume)</th>
<th>Number of extractions</th>
<th>Amount of reducing ketonic corticoids in urine number:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>25</td>
<td>1</td>
<td>54</td>
</tr>
<tr>
<td>25</td>
<td>2</td>
<td>65</td>
</tr>
<tr>
<td>25</td>
<td>3</td>
<td>58</td>
</tr>
<tr>
<td>25</td>
<td>4</td>
<td>59</td>
</tr>
<tr>
<td>25</td>
<td>5</td>
<td>63</td>
</tr>
<tr>
<td>50</td>
<td>2</td>
<td>59</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chloroform per cent (by volume)</th>
<th>Successive extraction</th>
<th>Urine number V</th>
<th>Ketonic fraction in 80 ml. of water (83 μg)</th>
<th>Cortin (§) 0.5 ml. in 80 ml. of water</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>I</td>
<td>12</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>50</td>
<td>II</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>100</td>
<td>I</td>
<td>25</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>II</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>III</td>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The effect of amount of chloroform and number of shakings on the extraction of reducing ketonic corticoids from urine and of ketonic corticoids and cortin added to water.

§) Cortine (Organon) = adrenal cortical extract.

The analysis can be interrupted at this stage and the neutral dry residue can be kept for a long time if stored in a refrigerator.

The recovery of desoxycorticosterone and 17-hydroxy-11-dehydrocorticosterone added to normal male urine and carried through the procedure described above is shown in Table 3.

GIRARD'S SEPARATION

In 1936 Girard & Sandulesco introduced the ketone-reagent, trimethyl acethyldrazide ammonium chloride (Girard reagent
### Table 3.

<table>
<thead>
<tr>
<th>Urine (pH 1)</th>
<th>DOC added</th>
<th>Cp. E added</th>
<th>Reducing value (DOC Standard)</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>ml</td>
<td>µg</td>
<td>µg</td>
<td>µg</td>
<td></td>
</tr>
<tr>
<td>A.</td>
<td>80</td>
<td>0</td>
<td>209</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>50</td>
<td>260</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>100</td>
<td>314</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>100</td>
<td>281</td>
<td>97*</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>200</td>
<td>358</td>
<td>202*</td>
</tr>
<tr>
<td>B.</td>
<td>80</td>
<td></td>
<td>580</td>
<td></td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>100</td>
<td>688</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>100</td>
<td>631</td>
<td>100×</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>200</td>
<td>676</td>
<td>188×</td>
</tr>
</tbody>
</table>


T) as a means of separating ketonic from non-ketonic steroids. This process, which has been of immense value for investigating the nature of steroid substances, is based upon the fact that, under certain conditions, ketonic steroids form water-soluble hydrazone derivatives with the reagent. These compounds are insoluble in organic lipoid solvents, and by extracting the mixture with ether or chloroform it is possible to remove the non-ketonic substances. The ketones can be liberated again from the hydrazone complex by acid hydrolysis and extracted afterwards with organic lipoid solvents.

**Girard & Sandulesco (1936)** dissolve the urinary extract in absolute alcohol. 10 per cent glacial acetic acid and 5—10 per cent reagent T are added. The mixture is heated on a boiling water bath for 30—60 minutes, and then cooled and transferred with ice-cold water to a separating funnel. Sufficient alkali is added to neutralize 90 per cent of the acetic acid and the non-ketonic steroids are immediately extracted with ether. The remaining watery solution is acidified to a 0.5 N concentration with HCl or H₂SO₄ and after standing for one hour
at room temperature, the ketonic steroids are extracted with ether.

Reichstein (1936) introduced some modifications, especially a fractional extraction of the ketonic steroids at different pH values, and used this method for the separation and purification of steroids from adrenal cortical extracts. He observed that the saturated steroids are easily liberated from the hydrazone complex by mild acid hydrolysis with weak acids, whilst unsaturated steroids, including the 3-α,β-unsaturated compounds, require strong acids and a longer time for hydrolysis.

Pincus & Pearlman (1941) worked out a micro-method for the separation of the 17-ketosteroids in small amounts of urine (24 hours samples). In this modification, the reaction is performed with the dry steroid to which 0.5 ml. glacial acetic acid and 100 mg. reagent T are added.

Talbot et al. (1945) used a similar method for fractionating the water-soluble corticoids obtained after the benzene-water partitioning of the crude urine extract.

Several other authors have used the original Girard method or modifications thereof (Talbot et al., 1940, Venning et al., 1944, Schiller et al., 1945, Dobriner et al., 1948). The main differences between the methods are in ratio of reagent to steroid, the temperature used during the process, the amounts of alkali and water, the time of hydrolysis of the hydrazones and the extraction procedures.

When the Girard process was adopted for a further fractionation of the corticoids for routine analyses, it obviously became necessary to determine the optimal conditions for the various steps in the process.

Amount of water and solvent: It was found that the process could be satisfactorily performed with small amounts (25 ml.) of water and lipoid solvents provided that the amount of steroid did not exceed 10 mg. and this micro-method has been used.

Dry steroid versus alcoholic solution: Several experiments were carried out with 10 mg. dehydroisoandrosterone (DHA) in a dry form or dissolved in 2 ml. alcohol. No significant dif-
ferences in the recoveries were obtained. We have therefore adopted the dry method, and the steroid is dissolved in only 0.8 ml. glacial acetic acid.

Amount of Girard reagent T: Most of the experiments were carried out with 10 mg. DHA and the quantity of reagent varied from 10 mg. to 200 mg. With 20 mg. of reagent or more the recoveries were maximal, i. e. about 95 per cent. In experiments with 111 µg cortisol the amounts of reagent were 12.5, 25, 50 and 100 mg. respectively, and the smallest quantity was found to be sufficient (97 per cent recovery in the ketonic fraction). In order to be certain that a sufficient amount of reagent is used when assaying urines with various and sometimes high corticoid contents, we use 25—40 mg. of reagent in the routine analyses.

Conditions for the hydrazone formation. Boiling versus room temperature: Girard & Sandulesco (1936) used boiling for 30—60 minutes; Talbot et al. (1940) heated the reaction mixture for 10 minutes on a boiling water-bath; Pincus & Pearlman (1941) warmed the mixture for 20 minutes at 90—100° C, while Venning et al. (1944) left the reaction mixture at room temperature for about 15 hours. Some of our own experiments with DHA, urinary extract of 17-ketosteroids, cortisol, DOC and a urinary extract of corticoids are summarized in Table 4.

As is evident from Table 4, almost complete recoveries are obtained after heating for 5 minutes or more on a boiling water-bath, or when the reaction mixture is left at room temperature overnight. The last-mentioned technique probably gives better and more consistent results. In the routine work it is an advantage if the analytical procedure can be interrupted at this stage and continued next morning.

Amount of alkali used for neutralization of the acetic acid: Most authors have adopted the original technique of Girard & Sandulesco and added sufficient alkali to the reaction mixture to neutralize 90 per cent of the acid. We have carried out several experiments with various amounts of NaOH and found that as soon as the pH is on the alkaline side, the recoveries were maximal, i. e. about 95 per cent. Before neutralization,
Table 4.

<table>
<thead>
<tr>
<th>Boiling water bath in minutes</th>
<th>Amount of corticoids recovered in ketonic fraction</th>
<th>urinary extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DHA (10 mg.)</td>
<td>Cortisone (111 µg)</td>
</tr>
<tr>
<td>2</td>
<td>7.65 (3)</td>
<td>103 (2)</td>
</tr>
<tr>
<td>5</td>
<td>9.67 (6)</td>
<td>105 (2)</td>
</tr>
<tr>
<td>10</td>
<td>9.60 (3)</td>
<td>100 (2)</td>
</tr>
<tr>
<td>15</td>
<td>9.34 (3)</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>9.20 (9)</td>
<td></td>
</tr>
<tr>
<td>Left at room temperature overnight</td>
<td>10.3 (2)</td>
<td>104 (5)</td>
</tr>
</tbody>
</table>

The effect of heating on boiling water bath, or of standing overnight at room temperature, on the hydrazone formation in the Girard process as measured by recoveries in the ketonic fraction. In addition to the results the figures in brackets also show the number of analyses performed.

The test-flask is cooled and the reaction mixture transferred to a separating funnel with 25 ml. of ice-cold water. It was, however, found that more consistent results can conveniently be obtained if the acid mixture is transferred with a dilute alkaline solution containing the required amount of NaOH. In the routine analyses this has been performed by the use of 25 ml. 0.55 N NaOH at 0° C. From the neutralized mixture the non-ketonic substances are then extracted by shaking 3 times with 25 ml. of ice-cold chloroform.

**Amount of acid necessary for the liberation of ketones from the hydrazone complex:** The amount of acid necessary depends on the aim of the process. When the Girard process is used as a means of identifying steroids, successive extractions at different pH values are performed. In clinical assays, in which a determination of the total amount of ketones is desired the extraction is always carried out at only one pH value. **Girard & Sandulesco** added HCl or H₂SO₄ in such amounts that a 0.5 N concentration was obtained. Different amounts of these

Downloaded from Bioscientifica.com at 11/16/2018 11:45:19AM via free access
two acids have been used by other investigators. Our own experimental data are given in Table 5. From the table it can be seen that consistent results can be obtained over a wide range of acid concentrations. Immediately after acidification 25 ml. chloroform are always added.

<table>
<thead>
<tr>
<th>Amount of 40 vol. per cent H₂SO₄ added for the acidification</th>
<th>DOC added</th>
<th>DOC recovered</th>
<th>Cortisone Acetate added</th>
<th>Cortisone Acetate recovered</th>
<th>Urinary extract L-52</th>
</tr>
</thead>
<tbody>
<tr>
<td>ml.</td>
<td>µg</td>
<td>µg</td>
<td>µg</td>
<td>µg</td>
<td>mg. per litre</td>
</tr>
<tr>
<td>1.0</td>
<td>100</td>
<td>89</td>
<td>200</td>
<td>200</td>
<td>0.30</td>
</tr>
<tr>
<td>1.5</td>
<td>100</td>
<td>92</td>
<td>200</td>
<td>200</td>
<td>0.44</td>
</tr>
<tr>
<td>2.0</td>
<td>100</td>
<td>93</td>
<td>200</td>
<td>184</td>
<td>0.44</td>
</tr>
<tr>
<td>2.5</td>
<td>100</td>
<td>95</td>
<td>200</td>
<td>189</td>
<td>0.44</td>
</tr>
<tr>
<td>3.0</td>
<td>100</td>
<td>93</td>
<td>200</td>
<td>184</td>
<td>0.39</td>
</tr>
<tr>
<td>3.5</td>
<td>100</td>
<td>98</td>
<td>200</td>
<td>184</td>
<td>0.42</td>
</tr>
</tbody>
</table>

The effect of the amount of added H₂SO₄ on the recovery of steroids in the ketonic fraction of the Girard process.

Some of the biologically active corticoids are sensitive to strong acids and *Heard, Sobel & Venning* (1946) found a marked loss in the reducing power of DOC and urinary corticoids after standing for 1 hour at room temperature in a medium containing 2 volumes per cent of sulphuric acid. In our experiments no significant loss was found even at the pH value of 0.4. It is our impression, however, that the free crystalline compounds and urinary corticoids, when dissolved in water, are very easily extracted by means of chloroform and this in all probability is explained by the fact that the liberated steroids rapidly pass from the acid aqueous phase to the lipid solvent.

In order to ensure a quantitative hydrolysis of the hydrazone complexes, we finally decided to use 2 ml. 40 vol. per cent H₂SO₄ which brings the pH value to just below 1.0. With this amount of acid the recoveries have been satisfactory in all cases.
After standing at room temperature for two hours, the ketonic fraction is extracted by shaking with the chloroform already present, and then further extracted twice with 25 ml. of chloroform.

The combined chloroform extracts are washed once with 10 ml. 0.1 N NaOH and three times with 10 ml. distilled water. Each washing is extracted back with 5 ml. chloroform as described above. After drying with anhydrous sodium sulphate and filtering, the chloroform extract is evaporated to dryness in an all-glass vacuum distillation apparatus at 45° C. The dry residue is then carefully transferred to a test-tube three times with 1 ml. of chloroform. After evaporation of the chloroform the test-tube can be stored in a refrigerator.

THE COLORIMETRIC ASSAY

To the dry residue two ml. of the phosphomolybdic acid reagent are added, the test-tube is closed with a cork wrapped with aluminium-foil and placed in a boiling water-bath for exactly 60 minutes as described by Heard & Sobel (1946). Immediately after cooling to room temperature, 8 ml. of the reagent are added and the mixture is transferred to a cuvette (internal diameter: 16 mm). The optical density is measured in a Coleman photometer (model 14) at 700 μm using a red filter (PC-5). The adjustment to zero optical density is performed with a reagent blank which is carried through the procedure mentioned above. The absorption curve for the molybdenum blue shows no maximum but a plateau from 685 μm to 800 μm.

Because of the difficulty in obtaining sufficient amounts of molybdic acid it has been necessary in most cases to use 50 per cent acetic acid for the dilution of the reagent mixture after the colour has been developed. Heard & Sobel (1946) found that this procedure can be used if the reading is per-
formed within 3 minutes, after which time the optical density decreases. We have been able to confirm this observation. It should be pointed out that the result is affected by the temperature at which the colour is developed. It is therefore very important to maintain uniform conditions for boiling. An electric hot plate or a steamboiler can be used.

Desoxycorticosterone is used as the standard of reference. A linear standard curve is found when the doses of DOC (e.g. 50, 100, 150, 200 μg) are plotted against the values for the optical density. From this the reducing equivalent of the urinary extract can be determined.

It has been necessary to control the standard curve for each new batch of reagent as it has been impossible to obtain uniform batches of molybdic acid.

AMOUNT OF CORTICOIDS IN THE URINE

It is not, as yet, possible to give the limits of the urinary excretion in normal human subjects but assays carried out on the urines from a small number of healthy subjects indicate that the values are of the order of 0.40 and 1.0 mg. per 24 hours. These values correspond rather closely with those obtained by estimating the formaldehyde liberating corticoids. The following results are mentioned in the literature: Heard, Sobel & Venning (1946): 1.0—2.0, Lowenstein et al. (1946): 0.5—0.8, Talbot et al. (1947): 0.10—0.44, Corcoran & Page (1948): average 1.0 mg. All these values represent the excretion per 24 hours in normal adults.

A patient suffering from Addison’s disease excreted 0.20 mg. per 24 hours in a state of crisis suggesting that an extra-adrenal source may produce some part of the reacting substances. On the same day the 17-ketosteroid excretion was found to be 0.5 mg, so that the presence of small remnants of functioning adrenal cortical tissue cannot be excluded.
EVALUATION OF THE METHOD

As the chemical test offers the advantages of speed and comparatively low variability (duplicate analyses are shown in Fig. 2), it is preferable to the troublesome and time con-

Fig. 2.
33 duplicate analyses plotted against each other.

suming procedure involved in biological tests. The available literature, however, reveals only a few attempts to correlate the values for the excretion of corticoids as determined by means of the chemical and biological methods, respectively, and hence some experiments were carried out on this question.

Shortly after the first reports from U.S.A. on the successful treatment of chronic rheumatoid arthritis with ACTH had reached Denmark, similar investigations were undertaken
here (Brochner-Mortensen et al., 1949). Several hundred 24-hour urines from ACTH treated patients have been analyzed for their corticoid content. We thus had ample opportunities for examining, firstly the agreement between the chemical and biological determinations, and secondly the correlation between the adrenal cortical function and the amounts of corticoids excreted.

24-hour specimens of urine were extracted in the usual manner and the total neutral fraction obtained was divided in aliquots for further processing, viz. chemical determination as described above and biological determination of the glucocorticoids by the method of Venning et al. (1946). Furthermore, in two cases a benzene-water partitioning was carried out, and the reducing substances obtained in the water fraction were colorimetrically determined as described above. Fig. 3 illustrates the data for the chemical assays in the two cases. In case J. D. there is a very close agreement of the absolute output values and in both cases there is a remarkably consistent correlation with regard to the relative output changes. Some time after the ACTH treatment, testosterone propionate was administered to one of the patients. During this period there was a marked increase in the excretion of 17-ketosteroids as determined by the micro-method of Hamburger (1948), but as can be seen, there was no effect on the excretion of corticoids, clearly indicating that the 17-ketosteroids do not interfere with the colorimetric assay of the corticoids. From these findings it is evident that no advantages is to be gained from a benzene-water partitioning of the urinary corticoids.

In Fig. 4 the results are shown of parallel chemical and biological determinations performed on urines from three patients, i.e. the two mentioned above and a third patient. The excretion curves for the biological determinations are more irregular during the ACTH treatment because of the greater variability in the bioassay. In all cases it is obvious that there is rather good agreement between the values obtained from day to day by the two methods. The percentage increase over the pre-treatment level is, however, more pronounced in the
Fig. 3.
The excretion of reducing ketonic corticoids and total water-soluble corticoids in two cases treated with ACTH. (Case J. D. above, and case L. R. below).

bioassay than in the chemical test. As an example, case A. R. may be considered. In this patient a hundred percent increase in the reducing corticoids was found while the increased excretion of biologically active glucocorticoids amounted to about 300 per cent.

This could be best explained on the assumption that the
Fig. 4.
The excretion of reducing ketonic corticoids and biologically active glucocorticoids in three cases treated with ACTH.

catabolism of the corticosteroids cannot keep pace with the greatly accelerated elaboration of these substances which occurs during ACTH-stimulation of the adrenal cortex. This results in a high titer of these biologically active corticosteroids in the blood, which in turn is reflected in a relatively higher excretion in the urine.

In addition, however, the possibility of a qualitative alteration in the composition of the urinary corticoids during the ACTH treatment must be kept in mind. If the regression line for the urinary glucocorticoids under these conditions acquires a different slope, as compared with the standard curve for cortisone, this should be considered as a part of the discrepancy observed. In this connection it is of interest to note that Olson et al. (1944), using a similar method, found similar qua-
litative differences in activity, when assaying several adrenal cortical extracts and the crystalline glucocorticoids. The problem will be further discussed in a later publication.

From several analyses carried out with urines from different patients and from normal human subjects it is clear that the absolute values for the diurnal excretion of reducing ketonic corticoids are consistently 8—10 times larger than those obtained by a simultaneous biological determination of the glucocorticoids. In the chemical assay, however, biologically active as well as biologically inactive metabolites are included and this fact must surely explain the difference observed. Very similar findings have been obtained in the chemical determination of 17-ketosteroids, as compared with the biological assay of the androgenic substances.

It can be concluded that all the neutral ketonic corticoids, as estimated by their reducing power, are metabolites closely related to the urinary biologically active glucocorticoids which are probably included among them. Furthermore it should be stressed that the chemical as well as the biological determinations reflect the cortical function in a similar manner.

**SUMMARY**

After a thorough experimental examination of the method of Heard, Sobel & Venning and of Talbot et al. for the determination of the urinary corticoids they were both abandoned. A method based on a combination of the principles used in the two methods is described. It is shown, however, that no advantage is gained by a benzene-water partition of the corticoids. An investigation on the different steps used in the analysis has been carried out in order to establish the optimal conditions.

The technical details in the procedure are outlined in the diagram below.

By means of simultaneous chemical and biological determinations on urines from ACTH treated patients, the relation between the reducing ketonic corticoids and the biological-
Adjust 1/10 of 24-hr. urine to pH 1 with 40 vol. per cent H₂SO₄
effect 3 times with re-distilled chloroform 25 per cent by volume

combine the chloroform extracts and wash 3 times by shaking with 10 ml. 0.1 N NaOH and 3 times with 10 ml. of water. Each washing is re-extracted with small amounts of chloroform.

dry the extract with Na₂SO₄, filter and evaporate to dryness = TOTAL NEUTRAL FRACTION
dissolve in 0.8 ml. glacial acetic acid add about 40 mg. Girard's reagent T
discard urine

leave the flask overnight at room temperature or heat the flask on boiling water-bath for 2 minutes

cool the flask in iced water

add 25 ml. ice cold 0.55 N NaOH and transfer to a separating funnel

extract immediately with ice cold chloroform, 3 times with 25 ml. (chloroform = non-ketonic fraction)
discard chloroform

add 2 ml. 40 vol. per cent H₂SO₄ and 25 ml. chloroform to the aqueous solution

2 hr. later extract with the chloroform already present and twice more with 25 ml. of chloroform

combine the chloroform extracts and wash by shaking 3 times with 10 ml. 0.1 N NaOH, and 3 times with 10 ml. of water re-extract each washing with small amounts of chloroform

dry the extract with Na₂SO₄, filter and evaporate to dryness = KETONIC FRACTION
ly active so-called glucocorticoids is shown. The agreement between the data is discussed. Both sets of results appear to reflect the adrenal cortical function in a similar manner.

ACKNOWLEDGEMENT

I am greatly indebted to Dr. J. Heer and Dr. A. Wettstein, Ciba, Basle, for the preparation of the free crystalline 17-hydroxy-11-dehydrocorticosterone, to Ciba Limited, Basle, for supplying me with desoxycorticosterone, and to Dr. Frederik Paulsen, Organon, Stockholm, for a Cortine preparation.

REFERENCES


