URINARY EXCRETION OF STEROIDS OF
ALLOPREGNANE-TETROL AND -PENTOL TYPES

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Fukushima et al. (1955) were the first, who have confirmed the excretion of steroids of allopregnane-tetrol and -pentol types in the urine of human being. They were able to isolate cortol, cortolone, β-cortol and β-cortolone from the urine after the administration of ACTH as well as a tracer dose of hydrocortisone-4-C¹⁴. Hitherto the view has widely supported that the major metabolites of hydrocortisone, the chief glucocorticoid in plasma of human being, are the glucuronides of their reduced ketols such as tetrahydro-E or -F, and a minor part is constituted of 11-oxygenated 17-ketosteroids.

How much the above steroids, the side chain of which is reduced, will be excreted, became now a focus of interest, on which, however, Fukushima gave no detailed information. In relating to the solution of this problem we have had much to do. The first was to extract those steroids with high polarities without any loss, and the second, to estimate them by a reaction, the most peculiar to those steroids.

The first problem was solved by continuous extraction of urine, treated with β-glucuronidase, with ethyl acetate and by purification of the extract by means of silica gel column chromatography, and the second problem, by quantitative estimation of 17-ketosteroids by Zimmermann reaction after the oxidation of eluate with periodic acid.

By those procedures the authors were able to estimate an approximate amount of allopregnane-tetrol and -pentol types, excreted daily in the urine. An investigation was also made on their excretion after the administration of ACTH or hydrocortisone.

EXPERIMENTAL PROCEDURES

Reagents
1) Chloroform : redistilled.
2) Ethanol: 5g of zinc powder and 5cc of 50% sodium hydroxide are added to 1000cc of absolute ethanol, refluxed to boiling for 30 mins. and redistilled.
3) Ethyl acetate: redistilled.
4) Acetate buffer: 0.2 M sodium acetate and 0.2 M acetic acid are mixed equally.

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5) Phenylhydrazine hydrochloride solution: 65 mg of phenylhydrazine hydrochloride, recrystallized from absolute ethanol, is dissolved in 100 cc of 50% sulfuric acid.

6) Chromotropic acid solution: 0.15 g of chromotropic acid is dissolved in 2 cc of distilled water and mixed with 48 cc of conc. sulfuric acid.

7) m-Dinitrobenzene solution: m-dinitrobenzene, chemically pure, is purified further according to the method of Drekter et al. (1947), and used as a 2% solution in ethanol.

8) β-Glucuronidase: the enzyme is produced from beef liver according to Bernfeld and Fishman’s method (1953). The purification is stopped at the third step of the procedure. The preparation is diluted with acetate buffer so as to contain 6000 Fishman units per cc.

9) Silica gel, pure; for chromatography.

10) 50% sulfuric acid.

11) 2.5 N Potassium hydroxide in ethanol solution: the solution is made freshly just before use.

12) Periodic acid solution: 0.6 g of potassium periodate is dissolved in 100 cc of 0.15 M sulfuric acid.

13) Stannous chloride solution: 0.3 g of stannous chloride is dissolved in 2 cc of hot conc. hydrochloric acid and diluted with 8 cc of distilled water. The solution is made freshly before use.

Methods

1) Extraction of steroids from the urine

10-20 cc of a freshly collected 24-hrs. urine specimen is adjusted to pH 4.5 with acetate buffer. β-Glucuronidase is added to this urine (300 Fishman units per cc of urine) thoroughly mixed and incubated at 47°C for 48 hrs.

The hydrolyzed urine is adjusted to pH 9.5 with sodium hydroxide solution and extracted with ethyl acetate for 24 hrs. by means of a continuous extracting apparatus. (Using an ordinary separating funnel no satisfactory result was obtained with any organic solvent in extracting the aimed steroids. The recoveries from an aqueous solution of cortolone (60 μg in 4 cc) were 50-78%. A continuous extraction with ether for 72 hrs. gave the recovery of 80%, and with ethyl acetate for 12 hrs. 90%.)

The ethyl acetate solution is evaporated to dryness, and the crude extract submitted to the column chromatography.

2) Silica gel column chromatography

500 mg of silica gel is put in a chromatographic tube, 10 mm in diameter and 20 cm long, and washed with 10 cc of 10% ethanol-chloroform, then with 10 cc of chloroform. The above extract is dissolved in 5 cc chloroform again and transferred quantitatively into the silica gel column. The column is eluted first with 20 cc of chloroform, then with 15 cc of 0.5% ethanol-chloroform and 25 cc of 1.0% ethanol-chloroform, 40 cc of 4.0% ethanol-chloroform and 25 cc of 10% ethanol-chloroform successively. The last two eluates were collected in each flask and evaporated to dryness. Porter-Silber chromogen and the steroids of pregnane-17,20-diol types (examined by acetaldehydegenetic method, Cox, 1952) are found in the 4% ethanol-chloroform fraction, and the steroids of allopregnane-tetrol and -pentol types in the 10% ethanol-chloroform fraction respectively.

3) The estimation of steroids of allopregnane-tetrol and -pentol types

The residue of 10% ethanol-chloroform eluate is dissolved in 0.1 cc of ethanol and diluted with 1 cc of water. 0.5 cc of periodic acid reagent is added to the solution and placed at room temperature for 30 mins. for oxidation. Excess oxidant then is removed by addition of 0.5 cc of stannous chloride solution, and extracted with 5 cc of chloroform three times. The chloroform solution is evaporated to dryness and the residue is submitted to the Zimmermann reaction by the method modified by Callow. (When the 10% ethanol-chloroform eluate was estimated as formaldehydegenetic corticosteroids, the value was always much higher than that of Zimmer-
mann chromogen. Perhaps this might be due to the contamination of the other formaldehydogenic organic acids in the extract. When the pH of the urine was made basic, pH 9.5, before the extraction, the value estimated by the formaldehydogenic method approached to that estimated by Zimmermann's reaction. The amount of Zimmermann chromogen is estimated by optical densities at the wave lengths of 460, 520 and 580 m\(\mu\), applying the following equation, proposed by Allen:

\[ \text{OD}_{520} - \frac{1}{2} (\text{OD}_{460} + \text{OD}_{580}) \]

The standard curve was made with androsterone and the estimated value as androsterone was multiplied by 1.27 on account of the difference of molecular weights between androsterone and cortolone.

4) The estimation of Porter-Silber chromogen

Porter-Silber chromogen in the 4% ethanol-choroform fraction of the hydrolyzed urine was estimated according to the modification of Glenn-Nelson's method (1954).

RESULTS

1) Daily urinary excretion of Porter-Silber chromogen and steroids of allo-pregnane-tetrol and -pentol types

The urinary excretion of steroids of allopregnane-tetrol and -pentol types in healthy adults was estimated by the above method, and the results are presented in Table 1. The values range from 3.4 to 10.0 mg per day in men and from 1.6 to 4.7 mg in women. It seems that they are a little higher in men than in women. Porter-Silber chromogen are accounted as 3.2-7.9 mg per day.

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Sex</th>
<th>Age</th>
<th>Porter-Silber chromogen (mg)</th>
<th>Allopregnane-tetrol &amp; -pentol (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>R. T.</td>
<td>m</td>
<td>28</td>
<td>5.5</td>
<td>10.0</td>
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<tr>
<td>2</td>
<td>Y. K.</td>
<td>m</td>
<td>29</td>
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<td>5.8</td>
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<tr>
<td>3</td>
<td>T. K.</td>
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<td>4.7</td>
<td>4.0</td>
</tr>
<tr>
<td>4</td>
<td>M. T.</td>
<td>m</td>
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<td>7.9</td>
<td>3.4</td>
</tr>
<tr>
<td>5</td>
<td>S. I.</td>
<td>m</td>
<td>26</td>
<td>5.3</td>
<td>7.6</td>
</tr>
<tr>
<td>6</td>
<td>H. A.</td>
<td>m</td>
<td>26</td>
<td>3.6</td>
<td>3.6</td>
</tr>
<tr>
<td>7</td>
<td>S. I.</td>
<td>f</td>
<td>23</td>
<td>3.3</td>
<td>3.0</td>
</tr>
<tr>
<td>8</td>
<td>M. K.</td>
<td>f</td>
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</tr>
<tr>
<td>9</td>
<td>K. A.</td>
<td>f</td>
<td>24</td>
<td>3.9</td>
<td>3.1</td>
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<tr>
<td>10</td>
<td>H. S.</td>
<td>f</td>
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<td>3.5</td>
<td>1.6</td>
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</table>

2) The effect of ACTH administration on the urinary excretion of Porter-Silber chromogen and steroids of allopregnane-tetrol and-pentol types

In two normal subjects, 25 mg of ACTH were injected intramuscularly and the effect was investigated on the urinary excretion of the above steroids. As shown in Table 2, the daily excretion of steroids of allopregnane-tetrol and -pentol types increased to 6.2-8.2 mg, about twice as much as the ordinary excretion (2.1-3.6 mg). Porter-Silber chromogen increased from the control level of 3.7-4.3 mg to 8.0-9.0 mg per day.
Table 2. The effect of ACTH administration (25 mg intramuscularly) on the urinary excretion of Porter-Silber chromogen and steroids of allopregnane-tetrol and -pentol types

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Sex</th>
<th>Age</th>
<th>Before</th>
<th>After the adm. of ACTH</th>
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<tbody>
<tr>
<td>1</td>
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<tr>
<td>2</td>
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<td>m</td>
<td>26</td>
<td>3.7</td>
<td>8.0</td>
</tr>
</tbody>
</table>

Table 3. The effect of per oral hydrocortisone administration on the urinary excretion of Porter-Silber chromogen and steroids of allopregnane-tetrol and -pentol types

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Sex</th>
<th>Age</th>
<th>Doses (mg)</th>
<th>Before</th>
<th>After the adm. of ACTH</th>
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</thead>
<tbody>
<tr>
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<td>m</td>
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<td>30.0</td>
<td>16.7</td>
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<td>2</td>
<td>T. M.</td>
<td>m</td>
<td>25</td>
<td>100</td>
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<td>13.8</td>
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<tr>
<td>3</td>
<td>M. T.</td>
<td>m</td>
<td>26</td>
<td>100</td>
<td>24.2</td>
<td>14.5</td>
</tr>
<tr>
<td>4</td>
<td>S. I.</td>
<td>m</td>
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<td>100</td>
<td>27.0</td>
<td>10.5</td>
</tr>
<tr>
<td>5</td>
<td>H. A.</td>
<td>m</td>
<td>26</td>
<td>100</td>
<td>23.0</td>
<td>10.9</td>
</tr>
<tr>
<td>6</td>
<td>K. A.</td>
<td>f</td>
<td>24</td>
<td>100</td>
<td>30.5</td>
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<tr>
<td>7</td>
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<td>m</td>
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<td>18.0</td>
</tr>
<tr>
<td>8</td>
<td>S. I.</td>
<td>f</td>
<td>23</td>
<td>50</td>
<td>8.2</td>
<td>18.7</td>
</tr>
<tr>
<td>9</td>
<td>M. K.</td>
<td>f</td>
<td>23</td>
<td>50</td>
<td>9.6</td>
<td>9.3</td>
</tr>
<tr>
<td>10</td>
<td>H. S.</td>
<td>f</td>
<td>26</td>
<td>50</td>
<td>10.4</td>
<td>14.0</td>
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</table>

DISCUSSION

It is already an established fact that hydrocortisone released from adrenal glands is converted ultimately to its reduced form of ring A, tetrahydro-F, which is then conjugated with glucuronic acid and excreted via the kidney into the urine. Then it was demonstrated of late by Fukushima et al. that steroids of tetrrol and pentol types, which are further reduced at the side chain, are likewise excreted in similar conjugated form. Those reduced steroids generally liberate one molecule of formaldehyde from one molecule of steroids of tetrrol and pentol types by the oxidation with periodic acid, but the steroids of tetrrol and pentol types give no more Porter-Silber reaction nor reducing reaction. Steroids of tetrrol and pentol
types, therefore, might be calculated as the so-called formaldehydogenic cortico-
steroids which is subtracted from Porter-Silber chromogen in a proper extract of
urine specimen; or the formaldehydogenic corticoids in far more selected fraction
(steroids of tetrol and pentol types) of urine might be attributable to the aimed
steroids. The above experiment, however, showed the incorrectness of the methods.

For the quantitative estimation of those steroids ethyl acetate had to be used
and the urine was extracted by means of a continuous extracting apparatus with
the solvent. The ethyl acetate extract contained much impurities, producing
formaldehyde by the oxidation with periodic acid, especially when the pH of urine
was made acid before extraction. The authors, therefore, rather selected the
indirect method for the determination of the aimed steroids by their conversion
into 17-ketosteroids (Tablot and Eitingon 1944) and applied this method to the
most polar fraction, chromatographed by the silica gel column, in which cortol and
cortolone should be eluated.

We estimated the amount of those steroids excreted in 24-urs. urine of nor-
mal adults in this way and 1.6-10.0 mg cortolone equivalent daily were account-
ed for. When 50-100 mg of hydrocortisone were administered orally to normal
adults, marked increases of steroids of those types were noted (10.5-37.4% of the
administered doses), as a proof that the latter certainly is a metabolites of the former.
On the other hand, we were able to recover 16.4-32.6% of administered hydro-
cortisone as Porter-Silber chromogen, when similar dose of hydrocortisone were
orally given.

So far as it has been observed in our laboratory 3% of administered hydro-
cortisone is excreted into the bile duct and 3-5% is oxidized to 11-oxygenated
17-ketosteroids, then all the amount of metabolites of administered hydrocortisone
sum up to about 41.9-62.2% of the administered doses. Therefore, 37.8-58.1% of
the administered doses remain unknown yet.

SUMMARY

The study was made on the quantitative estimation of steroids of allopregnane-
tetrol and -pentol types, excreted in urine.

1) The urinary excretion of the aimed steroids amounted to 1.6-10.0 mg per
day in healthy adults.

2) The administration of ACTH or hydrocortisone showed marked increases
in their daily excretion. When 50-100 mg hydrocortisone was administered, ca.
9.3-18.7 mg was excreted as steroids of allopregnane-tetrol and -pentol types and
8.2-32.6 mg as steroids of allopregnane-17,21-diol-20-one type respectively.

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REFERENCES