THE DETERMINATION OF FORMALDEHYDOGENIC STEROIDS IN URINE WITH A NOTE ON THE METABOLISM OF EXOGENOUS CORTISONE ACETATE

By

S. L. Tompsett and D. C. Smith

In a previous paper (Tompsett & Smith, 1955 a) it has been shown that the 21, 20, 17 triol steroid side chain is stable to the usual methods of hot acid hydrolysis. This observation was incorporated in a method for the determination of certain metabolites of cortisone present in urine. The products finally determined and known as the 17-ketogenic steroids (17. K. G. S.) were a measure of the 17-ketosteroids produced by periodic acid. Initially the urine was treated with sodium borohydride which reduced pre-formed 17-ketosteroids to non-reacting alcohols and the 21, 17 diol 20 one steroid side chain. The latter should be included in the final result.

The formation of 17-ketosteroids during the periodic acid reaction is accompanied by the liberation of formaldehyde. The determination of the formaldehyde liberated in this reaction forms the basis of the method described in this paper. This is, however, less specific, since formaldehyde but not 17-ketosteroids would be liberated from the corresponding 17-deoxy steroids. For this reason, studies with deoxycorticosterone have been included.

Initially the urine was treated with sodium borohydride, the function of which was to reduce 21, 17 diol 20 one to 21, 17, 20 triol and 21 ol 20 one 17 deoxy to 21, 20 diol 17 deoxy groups. Using pure steroids it was found that, based on the quantity of formaldehyde produced, only about 40 % of the steroid could be recovered by the chloroform extraction and washing processes employed. This figure, however, was consistent and similar over a wide range and was unaffected by previous treatment with hot dilute mineral acid. The result was not surprising in view of the large number of hydroxyl groups in the compounds being extracted. Details of further investigations into this problem have been recorded in another paper (Tompsett & Smith, 1955 d). Following reaction with periodic acid, it was found necessary
to separate the formaldehyde by distillation prior to its colorimetric determination with chromotropic acid -- 9 ml. of distillate were collected from a total volume of 15 ml. In contrast to acetaldehyde, formaldehyde cannot be completely recovered in this volume, and as a result it is necessary to carry out the distillation under standard conditions in order to obtain reproducible results (Smith & Tompsett, 1954). The colour developed during the chromotropic acid reaction is influenced by a number of factors (Smith, 1953) and a standard, cortisone or deoxycorticosterone, was therefore set up with each batch of periodate oxidations.

In the application of the method to urine, it has been decided to designate the material so measured as the formaldehydogenic steroids (F. S.). The figures reported for urine, refer to the actual quantity of cortisone derivative that may have been present originally. The excretion of F. S. in urine has been measured both before and after the oral administration of cortisone acetate. At the same time the 17. K. G. S. (Tompsett & Smith, 1955 a) were measured. To effect a comparison, the results were multiplied by two since it has been shown that cortisone produces approximately 50 % of such material in the procedure used. Also at the same time, a measure was made of the acid stable formaldehydogenic substance (A. S. F. S.) (Tompsett, 1953, Tompsett & Smith, 1954). The procedure was essentially the same as for F. S. except that preliminary treatment with sodium borohydride was excluded. The method of calculation was changed. Originally A. S. F. S. was expressed in terms of deoxycorticosterone and a 100 % recovery was assumed, as was generally obtained with this steroid. In this investigation, A. S. F. S. was calculated on the same basis as F. S., i.e. based on a final recovery of approximately 40 % of steroid.

R E A G E N T S

1. Cortisone (Organon Ltd.) in ethanol 1 mg. per ml.
2. Deoxycorticosterone (Organon Ltd.) in ethanol 1 mg. per ml.
3. Sodium Borohydride (L. Light & Co.).
5. 10 % (w/v) Potassium dihydrogen phosphate.
6. M/150 Phosphate buffer, pH 6.5.
8. Periodate Reagent – 0.01 M potassium periodate in 0.15 M sulphuric acid.
9. 9 M. Sulphuric acid.
10. 2 N. Aqueous sodium hydroxide.
12. Chromotropic acid reagent – prepared fresh before use.
   0.2 gm. of chromotropic acid (specially purified) was dissolved in 2 ml. of water and 48 ml. of 13 M sulphuric acid added.
EXPERIMENTAL

1. Cortisone or deoxycorticosterone was reduced with sodium borohydride. A chloroform extract was prepared. After removal of the chloroform, the residue was allowed to react with periodic acid. The formaldehyde liberated, was measured colorimetrically with the chromotropic acid reagent, after its separation by distillation. Hot acid hydrolysis was not employed.

0.5, 1.0 or 2.0 mg. of steroid was added to 100 ml. of M/150 phosphate buffer solution, pH 6.5. 0.4 gm. of sodium borohydride was added and the mixture stirred at intervals. The pH was kept below 8 by the addition of 10 % potassium dihydrogen phosphate solution. At the end of 15 minutes, 20 ml. of concentrated hydrochloric acid were added and the mixture allowed to stand 10 minutes to complete the destruction of borohydride.

The mixture was extracted three times with 50 ml. quantities of chloroform. The combined chloroform extracts were washed once with 25 ml. of 2 N aqueous sodium hydroxide and twice with 25 ml. quantities of water. Excess of anhydrous sodium sulphate was added to the chloroform extract to dehydrate. After 30 minutes the mixture was filtered and the residue washed with further quantities of chloroform. The chloroform extract was evaporated to dryness in an all glass distillation apparatus.

The residue was dissolved in 1 ml. of ethanol, 5 ml. periodate reagent added and the mixture allowed to stand overnight. At the same time, a blank, and a standard containing 1 mg. of the steroid under investigation, were set up. Then 0.5 gm. of stannous chloride was added to neutralise the periodate. The mixture was transferred to a 50 ml. flask attached to a water cooled condenser together with 0.5 ml. of concentrated sulphuric acid and sufficient water to a total volume of 15 ml. The mixture was heated to boiling and the distillate collected in a tube containing 1 ml. of water. Distillation was continued until a total volume of 10 ml. were obtained.

Colorimetric Determination of Formaldehyde:

Into a test tube were measured, 1 ml. of distillate, 2 ml. of water and 5 ml. of chromotropic acid reagent. After mixing, the tubes were placed in a boiling water bath for 30 minutes. After cooling, the volumes were adjusted to 10 ml. by the addition of 13 M sulphuric acid. Readings were made against the blank in a Unicam Spectrophotometer S. P. 350 at 565 m\u03bc.

Typical results are shown in Table 1. The results are expressed as a percentage of the formaldehyde determined in comparison with that obtainable from the original steroid. Recoveries were apparently low but consistent and similar over a wide range.

2. The experiments described above were repeated but hot acid hydrolysis was included. Following treatment with sodium borohydride, 20 ml. of con-
Table 1.
The recovery of deoxycorticosterone and cortisone after treatment with sodium borohydride. The recovery is expressed in terms of the % formaldehyde determined on reaction with periodic acid in comparison with the untreated steroid.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Micrograms used</th>
<th>/% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Without Acid Hydrolysis</td>
</tr>
<tr>
<td>Deoxycorticosterone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ditto</td>
<td>500</td>
<td>45.3</td>
</tr>
<tr>
<td>ditto</td>
<td>1000</td>
<td>44.2</td>
</tr>
<tr>
<td>ditto</td>
<td>2000</td>
<td>43.9</td>
</tr>
<tr>
<td>Cortisone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ditto</td>
<td>500</td>
<td>44.1</td>
</tr>
<tr>
<td>ditto</td>
<td>1000</td>
<td>43.8</td>
</tr>
<tr>
<td>ditto</td>
<td>2000</td>
<td>42.3</td>
</tr>
</tbody>
</table>

Table 2.
The recovery of deoxycorticosterone and cortisone added to urine. Determined as Formaldehydogenic Steroids (F.S.) – after treatment with Sodium Borohydride, the formaldehyde liberated on reaction with periodic acid was measured. The results were calculated from a curve determined from deoxycorticosterone or cortisone that had been treated similarly. – Volume of urine used – 100 ml. – The results are expressed in terms of micrograms F.S.

<table>
<thead>
<tr>
<th>Initial Content</th>
<th>Steroid added μg.</th>
<th>Total F. S. measured</th>
<th>Added F. S. recovered</th>
<th>/% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deoxycorticosterone</td>
<td>900</td>
<td>500</td>
<td>1450</td>
<td>550</td>
</tr>
<tr>
<td></td>
<td>900</td>
<td>1000</td>
<td>1975</td>
<td>1075</td>
</tr>
<tr>
<td></td>
<td>900</td>
<td>2000</td>
<td>3025</td>
<td>2125</td>
</tr>
<tr>
<td></td>
<td>1375</td>
<td>500</td>
<td>1850</td>
<td>475</td>
</tr>
<tr>
<td></td>
<td>1375</td>
<td>1000</td>
<td>2450</td>
<td>1075</td>
</tr>
<tr>
<td></td>
<td>1375</td>
<td>2000</td>
<td>3300</td>
<td>1925</td>
</tr>
<tr>
<td>Cortisone</td>
<td>875</td>
<td>500</td>
<td>1400</td>
<td>525</td>
</tr>
<tr>
<td></td>
<td>875</td>
<td>1000</td>
<td>1950</td>
<td>1075</td>
</tr>
<tr>
<td></td>
<td>875</td>
<td>2000</td>
<td>2700</td>
<td>1825</td>
</tr>
<tr>
<td></td>
<td>925</td>
<td>500</td>
<td>1400</td>
<td>475</td>
</tr>
<tr>
<td></td>
<td>925</td>
<td>1000</td>
<td>1950</td>
<td>1025</td>
</tr>
<tr>
<td></td>
<td>925</td>
<td>2000</td>
<td>2950</td>
<td>2025</td>
</tr>
</tbody>
</table>

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centrated hydrochloric acid were added and the mixture boiled under a reflux condenser for 10 minutes. The mixture was cooled and the procedure continued as described above.

Typical results are shown in Table 1. It will be noted that the inclusion of a hot acid hydrolytic procedure had no appreciable effect upon the quantity of formaldehyde determined.

3. The procedure was then applied to urine as described by Tompsett & Smith (1955 a), i.e. hot acid hydrolysis was included. 100 ml. of urine was used on each occasion. The recovery of cortisone and deoxycorticosterone added to urine was examined. 0.5, 1.0 or 2.0 mg. of steroid was added to 100 ml. of urine and the complete procedure carried out.

Calculations were based on a curve derived by the procedure described by Tompsett & Smith (1955 a). From the results shown in Table 2, it will be seen that reasonable recovery of both steroids could be obtained from urine.

4. Urine was then examined both before and after the oral administration of cortisone acetate. The following determinations were carried out: –

| Table 3. |
The effect of the oral administration of cortisone acetate upon the excretion of formaldehydogenic steroids (F. S.) in urine. |
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>A. S. F. S.</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>1. Control after 100 mg. cortisone acetate</td>
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<tr>
<td>2. Control after 150 mg. cortisone acetate</td>
</tr>
<tr>
<td>3. Control after 200 mg. cortisone acetate</td>
</tr>
</tbody>
</table>

1) A. without borohydride treatment referred to in the text as A. S. F. S.

B. borohydride treatment included referred to in the text as F. S.

2) it has been assumed that the total F. S. excreted is derived from exogenous sources.

3) 17-ketogenic steroids.
A. S. F. S., F. S., and 17. K. G. S., and the results are shown in Table 3. Calculations for A. S. F. S. and F. S. were based on a curve derived from cortisone by the procedure described by Tompsett & Smith (1955 a).

It will be noted that:

(a) about 40% of the dose of cortisone acetate could be recovered in urine in the form of F. S. within the first 24 hours.
(b) in control urines and urines collected after the administration of cortisone acetate, F. S. and the cortisone equivalents of the 17. K. G. S. (17. K. G. S. × 2) are of the same order,
(c) the effect of administered cortisone acetate
   (i) exerts a greater effect upon the excretion of F. S. than of A. S. F. S.
   (ii) the ratio A. S. F. S./F. S. is considerably less after oral cortisone acetate administration.

DISCUSSION

It must be emphasised that the procedure described is experimental. The determination of formaldehyde liberated by periodic acid is a non-specific reaction and many non-steroidal substances present in urine react similarly (Tompsett & Smith, 1953, 1954). It is believed, however, that the preliminary processes do achieve the necessary degree or specificity and hence the formaldehyde finally measured is of steroidal origin.

To achieve reproducibility greater care appears to be necessary than in the case of 17. K. G. S. This is particularly necessary at two stages (1) the extraction processes with chloroform followed by the necessary washings and (2) the separation of formaldehyde by distillation. To obtain reproducible results by chloroform extraction, it is necessary to obtain clear separations. Separation as emulsions would certainly invalidate any correction values that may be used. Separation by centrifuging is necessary in such cases. Provided attention has been paid to detail, the determination of F. S. probably gives a more accurate estimate than that of 17. K. G. S., especially at low concentrations, since colour corrections are unnecessary.

The determination of F. S. will include 17-deoxy steroid derivatives related to corticosterone. Evidence to date would suggest that the major steroid secreted by the human adrenal steroid is cortisone or a closely related steroid, corticosterone being secreted in insignificant quantities (Bush & Sandberg, 1953). In an examination of human blood steroids these authors have encountered corticosterone as the major steroid on rare occasions. A comparison of the results obtained by the two methods, F. S. and 17. K. G. S., may prove of some value. In the present small series of control urines, the two values are so close as to suggest only insignificant amounts of 17-deoxy steroid. A large
positive difference between F. S. and 17. K. G. S. would be suggestive of the presence of appreciable quantities of corticosterone.

A procedure has been described for the determination of what have been designated as the acid stable formaldehydogenic steroids (A. S. F. S.) of urine (Tompsett, 1953, Tompsett & Smith, 1954). The procedure was essentially the same as for F. S. except that treatment with sodium borohydride was not included. Usually marked increases in excretion were encountered after treatment with corticotrophin. In earlier work, the administration of cortisone acetate had but slight effect. In later work appreciable increases were noted especially after high dosages. It had been shown (Tompsett, 1953) that the side chain characteristic of deoxycorticosterone was stable to the hot acid hydrolysis procedure employed and this has been confirmed (Marrian, Paterson & Atherden, 1953). It was therefore considered that A. S. F. S. represented a measure of 17-deoxysteroid secretion. With regard to the stability of the cortisol side chain, there is some doubt, but fortunately does not require real consideration in this paper. The results shown in Table 3 may, however, help to elucidate this problem, which of course will require much more detailed investigation. The closeness of the values for F. S. and 17. K. G. S. (× 2) suggest that the urinary extracts contained but little 17-deoxysteroid. The suggestion is made that in all probability, except in some cases, A. S. F. S. is a measure of cortisol metabolites with a 21, 20, 17 triol side chain, whilst F. S. includes metabolites with a 21, 17 diol 20 one side chain.

The control urines all showed A. S. F. S./F. S. values of approximately 70% which would suggest that the greater part of the excreted cortisol metabolites have the 21, 20, 17 triol side chain. Orally administered cortisol acetate appears to be excreted in a much higher percentage as a metabolite with a 21, 17 diol 20 one side chain. This could account for the slight response in A. S. F. S. excretion reported in earlier experiments following the administration of cortisol acetate.

It has been customary to base steroid secretion on the measurement of one type of metabolite, e.g. 17-ketosteroids, pregnanediol, A. S. F. S., 17, 21 dihydroxy corticosteroids (Porter-Silber Reaction). It would appear from the results reported in this paper that due to changes in metabolic pathway, erroneous interpretations might be arrived at.

A number of writers have measured the non-ketonic alcohols present in the neutral steroid extract of urine (Tompsett & Oastler, 1948, Tompsett, 1951, Nathanson, Engel & Kelley, 1951). Tompsett & Oastler noted a marked increase in non-ketonic alcohols following surgical trauma while Nathanson et al. noted that the increase in this fraction was the most consistent feature of corticotrophin therapy. The nature of these substances has never been characterised but there is the possibility that they may be mainly related to the A. S. F. S. fraction.
SUMMARY

It has been confirmed that the steroid group, 21, 20, 17 triol, is stable to hot dilute mineral acids as used in the usual hydrolytic procedures. This observation has been utilised in a method for the determination of cortisone metabolites in urine. The urine is treated with sodium borohydride and then the steroids are liberated from their conjugates by boiling with hot dilute mineral acid. The formaldehydogenic steroids (F.S.) are determined by measuring the formaldehyde liberated by periodic acid. After oral administration of cortisone acetate, about 40% of the dose could be recovered in urine during the first 24 hours. In the small series of urines examined, a comparison of F.S. and 17.K.G.S. values would suggest that very little 17-deoxysteroid is present in normal urine.

REFERENCES

Tompsett, S. L.: J. clin. Path. 6, 74, 1953.