URINARY METABOLITES OF ADMINISTERED CORTICOSTERONE

I. STEROIDS LIBERATED BY GLUCURONIDASE HYDROLYSIS*

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While a number of urinary steroids related to the principal human adrenal secretory product, hydrocortisone, are known, little information is available regarding the metabolic fate of corticosterone. Mason (1) has isolated pregnane-3α,20α-diol-11-one from the urine of patients treated with dehydrocorticosterone. Lieberman, Fukushima, and Dobriner (2) have also isolated this substance, as well as pregnane-3α,ol-11,20-dione from human urine. In a recent publication, Gómez Mont and Berliner (3) reported the detection of corticosterone and an incompletely characterized tetrahydro derivative of dehydrocorticosterone in the urine of a patient treated with corticosterone. This report deals with the isolation and identification of allopregnane-3α,11β,21-triol-20-one, pregnane-3α,11β,21-triol-20-one, pregnane-3α,21-diol-11,20-dione, and pregnane-3α,20α-diol-11-one from the urine of a patient with rheumatoid arthritis who was treated with 300 mg. of corticosterone per day for 8 days.

Methods

Hydrolysis of Conjugates and Preparation of Extracts—One-tenth of each 24 hour specimen (equivalent to a total of eight-tenths of a 24 hour specimen) was boiled for 10 minutes (1), cooled, adjusted to pH 5.1 with acetate buffer, and treated with 30,000 Fishman units of beef liver β-glucuronidase (Warner). After incubation at 37° for 3 days, the hydrolysis mixture was extracted with methylene chloride and washed with saturated sodium bicarbonate solution and water. The residues obtained from these extracts by evaporation under reduced pressure were dissolved in ethanol.

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Assay of Formaldehydogenic Lipide—Aliquots were withdrawn from ethanol solutions of the extracts and analyzed for formaldehydogenic lipides by a modification of the method of Hollander et al. (5). The ethanol solution of the sample was transferred into the outer well of a Conway unit and the solvent allowed to evaporate spontaneously. The residue was dissolved in 0.5 ml. of triethyl phosphate. 2 ml. of 0.30 per cent chromotropic acid in 15 M sulfuric acid were placed in the inner well, and finally 1 ml. of 0.02 M aqueous sodium periodate solution in 0.15 M H$_2$SO$_4$ was added to the outer well. The units were then sealed and oxidation and diffusion were allowed to proceed at room temperature in the dark for 16 hours. The contents of the inner wells were then transferred to 13 X 100 mm. Pyrex test-tubes calibrated for use as cuvettes, heated on the boiling water bath for 30 minutes, cooled, and the absorbance at 570 m$\mu$ measured in a Coleman junior spectrophotometer.

Separation of Ketonic and Non-Ketonic Fractions—This separation was accomplished in the usual manner with Girard's Reagent T (6). The reaction was carried out at room temperature for 24 hours, and all extractions were made with chloroform.

Counter-Current Distributions—A 100 tube glass instrument (7) was used for all distributions. The solvents were mutually saturated, and the system (50 per cent cyclohexane-50 per cent ethyl acetate/30 per cent ethanol-70 per cent water) used was selected so that hydrocortisone would have $K = 1.1$ (8). Analyses for formaldehydogenic lipides were performed on selected fractions.

Paper Chromatography—Fractions and appropriate standards were applied to 7 inch strips of Whatman No. 1 filter paper and chromatographed in the toluene-propylene glycol system of Burton, Zaffaroni, and Keutmann (9). The zones were detected by examination under ultraviolet light and by spraying test strips with alkaline blue tetrazolium. The steroids were eluted with methanol, and the extracts evaporated to dryness, dissolved in chloroform, and washed with small volumes of water to remove propylene glycol. Prior to examination in the infra-red, the fractions eluted from paper chromatograms were further purified. The samples were dissolved in a small volume of cyclohexane-ethyl acetate and applied to a 7 X 150 mm. column of silica gel (Davison, 200 to 300 mesh). Elution was carried out with cyclohexane containing increasing amounts of ethyl acetate and finally with ethyl acetate.

Acetylation—The sample was dissolved in 0.1 ml. of pyridine (distilled from barium oxide) and treated with 0.1 ml. of redistilled acetic anhydride. After standing overnight at room temperature, the volatile reagents were removed at about 60° in a stream of air. The last traces were eliminated by storage overnight in a vacuum desiccator over silica gel.
Chromic Acid Oxidation—Chromic acid oxidation was carried out as described by Zaffaroni and Burton (10).

Preparation of Methyl Alloetianates—The sample was dissolved in 1 ml. of redistilled methanol and treated with 4 per cent periodic acid in 0.2 N H₂SO₄ and allowed to stand at room temperature overnight. The reaction mixture was then diluted with 10 ml. of water and extracted with five 10 ml. portions of chloroform and the extract washed with five 5 ml. por-

![Image of fractionation of urine for the isolation of corticosterone metabolites](http://www.jbc.org/)

**Table I**

**Paper Chromatography of Ketonic Fraction**

<table>
<thead>
<tr>
<th>Zone No.*</th>
<th>Blue tetrazolium</th>
<th>Formaldehydogenic lipides †</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>0.3</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>1.1</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>2.3</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>13.2</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>5.6</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>0.1</td>
</tr>
</tbody>
</table>

* The zones are listed in order of decreasing mobility.
† Calculated as mg. of deoxycorticosterone.

Injra-Red Spectra—A Perkin-Elmer double beam infra-red spectrometer

1 Obtained from the Aldrich Chemical Company, Inc., Milwaukee, Wisconsin.
was used for all measurements. Unless otherwise indicated, the spectra were measured in carbon disulfide solution.

Results

The procedure employed for processing the urine is outlined in the flow sheet (Fig. 1). Both the ketonic and the non-ketonic fractions were subjected to 150 transfer counter-current distributions.

Analysis of the distribution of the ketonic fraction for formaldehydogenic lipides disclosed that the bulk of the material was contained in one broad band which extended from tubes 90 to 99 in the instrument and transfer numbers 150 to 110 in the portion withdrawn. Such a band spread was considerably greater than would be predicted for a single compound. Accordingly, this material was pooled and subjected to further fractionation by paper chromatography.

The results of paper chromatography are presented in Table I. Of the six zones observed, only three contained sufficient material for characterization.

Characterization of Zone 4—Examination of this material in the infra-red showed strong hydroxyl absorption and absorption in the carbonyl region strongly suggestive of the presence of 3-, 11-, or 20-carbonyl groups. Acetylation yielded a substance whose infra-red absorption was identical in every respect with that of an authentic specimen of pregnane-3α,21-diol-11,20-dione 3,21-diacetate (I).

Characterization of Zone 5—The washed residue from Zone 5 crystallized spontaneously. After further purification by four crystallizations from aqueous methanol, 5 mg. of pure compound, m.p. 201–206° (uncorrected, Fisher-Johns) were obtained. This substance did not form an insoluble digitonide. Analysis for acylable hydroxyl groups (12) showed 1.8 hydroxyls for a molecular weight corresponding to a C_{21}O_{4} structure.

The free compound was too sparingly soluble in either carbon disulfide or chloroform to yield satisfactory infra-red spectra. However, the diacetate had strong hydroxyl absorption and, in addition, bands at 1757 and 1735 cm.⁻¹, which indicated a 20-keto-21-acetoxy structure (13). This was in agreement with the observation that the free compound strongly reduced alkaline blue tetrazolium.

Chromic acid oxidation of the diacetate yielded a material which no
longer had hydroxyl absorption in the high frequency region. However, it retained its absorption at 1755 and 1735 cm$^{-1}$ and possessed, in addition, a new band at 1711 cm$^{-1}$. The presence of a hydroxyl group not susceptible to acetylation under conditions of basic catalysis and its ready oxidation to a ketone group absorbing at 1711 cm$^{-1}$ make it apparent that the original compound must have contained an $11\beta$-hydroxyl group.

Since the spectrum of the 11-ketodiacetate in the 1350 to 750 cm$^{-1}$ region was distinctly different from that of pregnane-3$\alpha$,21-diol-11,20-dione diacetate (I) and Compound 5 did not form an insoluble digitonide, it was tentatively assigned the structure allopregnane-3$\alpha$,11$\beta$,21-triol-20-one (II).

Since no reference standard was available, it was necessary to degrade the compound to a known alloetianic acid. A parallel degradation was carried out with allopregnane-3$\beta$,11$\beta$,21-triol-20-one (Reichstein's Substance R) (III) (14, 15). The two methyl esters (IV and V) had distinctly different absorption in the 1350 to 750 cm$^{-1}$ region, but both had similar strong bands in the carbonyl region characteristic of carbomethoxy groups. The methyl alloetianate (V) derived from Substance R had its maximum at 1738 cm$^{-1}$, while that from Compound 5 (IV) showed maximal absorption at 1736 cm$^{-1}$.

Chromic acid oxidation of the two methyl esters yielded substances which had identical infra-red spectra throughout the range usually measured. The degradation path from III makes it apparent that VI must be methyl 3,11-diketoalloetianate.

Further support for this structure was obtained by examination of the
infra-red spectra of the acetates VII and VIII. Acetate VII had the complex acetate absorption (1256, 1237 cm\(^{-1}\)) characteristic of the axial conformation (16) (3α,5α or 3β,5β), while VIII, which has the equatorial conformation (3β,5α or 3α,5β), had a single band at 1240 cm\(^{-1}\). Since Compound 5 does not form an insoluble digitonide and has been degraded to an alloetianic acid, there can be little doubt that it is allopregnane-3α,11β,21-triol-20-one (II).\(^2\)

**Characterization of Zone 6**—The eluate from Zone 6 was further purified by passage through a column of silica gel. Examination of the eluate in the infra-red revealed strong hydroxyl absorption and a band at 1708 cm\(^{-1}\) (20-ketone). Acetylation yielded a substance which still had hydroxyl absorption and, in addition, bands at 1755 and 1734 cm\(^{-1}\) characteristic of a 20-keto-21-acetoxy structure. Chromic acid oxidation of the acetylated material yielded a substance the infra-red absorption of which was identical in every respect with that of the authentic sample of I and the diacetate of Compound 4. Compound 6 is therefore pregnane-3α,11β,21-triol-20-one and its diacetate is IX.

**Non-Ketonic Fraction**—The total non-ketonic portion of the formaldehydogenic lipides was subjected to a 150 transfer counter-current distribution, and selected tubes were analyzed for formaldehydogenic lipides and acylable lipides. The principal acylable lipide was found at transfer numbers 130 to 110. A small amount of formaldehydogenic lipide was also present in this region. The contents of these tubes were combined, oxidized with periodic acid, and the neutral oxidation products freed from carbonyl compounds with the aid of Girard's Reagent T. After acetylation, the infra-red spectrum of the non-ketonic fraction was indistinguishable from that of an authentic specimen of pregnane-3α,20α-diol-11-one diacetate.

**DISCUSSION**

Counter-current distribution and paper chromatography were used as complementary techniques for the resolution of the steroid mixture isolated from urine after β-glucuronidase hydrolysis. While the number of transfers applied was insufficient to separate the individual components, the character of the curve provided evidence for the presence of more than one component in the major band. In the case of the non-ketonic fraction, the application of two analytical parameters revealed the presence of a formaldehydogenic substance which contaminated the major alcoholic component.

\(^2\) Direct comparison of the infra-red spectrum of Compound 5 diacetate with that of a sample of allopregnane-3α,11β,21-triol-20-one 3,21-diacetate prepared by partial synthesis completely established their identity. The synthetic material was generously sent us by Dr. F. C. Dohan.
Subsequently, paper chromatography was used to resolve the major components of the ketonic fraction, three of which were identified, two by direct comparison with authentic specimens and the third by degradation to a known compound. Since the quantity of material was insufficient for characterization of intermediates and the final product by classical procedures, the structural conclusions were based almost entirely upon spectral evidence. However, the chemical evidence and spectral evidence are wholly consistent with one another.

It is noteworthy that the principal \( \alpha \)-ketol isolated from the urine of this patient during the administration of corticosterone should belong to the \( 3\alpha,5\alpha \) series (Table II). This is in contrast to the metabolites of progesterone, deoxycorticosterone, cortisone, and hydrocortisone whose major metabolites belong to the \( 3\alpha,5\beta \) series (17). Whether this departure from the usual pattern is structurally determined or is a metabolic characteristic of the particular patient or of his disease cannot be ascertained from this experiment. It should be mentioned that for the compounds thus far isolated the ratio of \( 5\beta \) to \( 5\alpha \) steroids is 1.1.

We wish to thank Dr. Walter Bauer and Dr. William S. Clark for making available the urine specimens, Dr. L. H. Sarett for his gift of pregnane-\( 3\alpha,21\)-dial-11, 20-dione, and Dr. A. Zaffaroni for his gift of allopregnane-\( 3\beta,11\beta,21\)-triol-20-one (Reichstein’s Substance R). Our thanks are also due Miss Marjorie J. Springer and Miss Faiza A. Fawaz for measuring the infra-red spectra.

**SUMMARY**

Allopregnane-\( 3\alpha,11\beta,21\)-triol-20-one, pregnane-\( 3\alpha,11\beta,21\)-triol-11-one, pregnane-\( 3\alpha,21\)-dial-11, 20-dione, and pregnane-\( 3\alpha,20\alpha\)-dial-11-one have been isolated from the urine of a patient treated with corticosterone.
BIBLIOGRAPHY

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STEROIDS LIBERATED BY GLUCURONIDASE HYDROLYSIS
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