STEROID ISOLATION STUDIES IN CONGENITAL ADRENAL HYPERPLASIA*

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Recent synthetic studies of the preparation of 17α,20α-dihydroxy-steroids from 17α-hydroxy-20-ketosteroids and 17α,20β-epoxy-20α-acetoxy-steroids have furnished hitherto undescribed compounds for comparison with "non-ketonic" urinary components (1). With these reference compounds in hand, two new metabolites were identified from urine of a patient with congenital adrenal hyperplasia. These compounds were allo-pregnan-3α,17α,20α-triol (I) and pregnane-3α,11-17β,20α-tetrol (II). A third component was obtained, 11-ketopregnane-3α,17α,20α-triol (III), previously isolated and characterized by Finkelstein, von Euw, and Reichstein (2). A fourth glycol, allopregnan-3α,17α,20β-triol, was identified by infrared spectrometry and paper chromatography but was present in insufficient amount for isolation in the conventional manner.¹ Large amounts of other neutral steroids were found in this urine after enzymic hydrolysis. These are listed in Table I.

Derivatives of allopregnan with a hydroxyl group in the α orientation at C-3 have previously been isolated from human urine; their precursors, however, have been steroid hormones, such as progesterone and corticosterone, which lacked the 17α-hydroxyl group. The presence in urine of the 3α-hydroxylallopregnan compounds described in this investigation demonstrates that the Δ^4-3-keto group of 17-hydroxylated adrenal hormones can be reduced in vivo to saturated alcohols of this type. It is therefore highly probable that 3α-hydroxyallopregnan derivatives are metabolites of hydrocortisone and will be found in urine; the synthesis of these suspected end products is in progress.

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¹ The isolation of 3α,17α-dihydroxyallopregnane-20-one from the urine of this patient was previously reported (3).
A 6 day collection of urine of a man with congenital adrenal hyperplasia was treated with 3-glucuronidase at pH 5 and 37° for 5 days. The urine was adjusted to pH 1 and was continuously extracted with ether for 48 hours. The neutral fraction was obtained in the usual way. The residual urine and the alkaline washes from the initial extract were combined and were acidified to 1 N sulfuric acid. After continuous ether extraction for 48 hours, the neutral fraction was prepared as before. The individual neutral fractions were separated into ketonic and non-ketonic fractions by means of Girard’s Reagent T and the former was separated into α- and β-ketosteroid subfractions with digitonin. A portion of each subfraction was chromatographed on paper; the results are reported in Table I.

The non-ketonic fraction from the enzyme-hydrolyzed portion only was submitted to a series of chromatographic separations with use of the partition systems described by Katzenellenbogen and coworkers (4). Various eluates were examined by infrared spectrometry and, where individual components or simple mixtures were recognized, the appropriate eluates were combined and purified. More complex mixtures or portions of the chromatogram which contained material with a distinctive but

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\* All melting points are corrected.
\* We thank our colleagues Dr. Olaf Pearson and Dr. Willet F. Whitmore for making this patient’s urine available for the study. A description of the clinical studies will be reported elsewhere.

\* The enzyme, 3-glucuronidase, used for hydrolysis was obtained from the Warner-Chilcott Laboratories, a division of Warner-Lambert Pharmaceutical Company, New York; it is commercially available under the trade name Ketodase.
unrecognized spectrum were combined and rechromatographed until the infrared spectrum was only slightly altered by further fractionation. In addition, selected areas of the chromatogram were examined by partition chromatography on paper for evidence of homogeneity of the constituents. In all, eight chromatograms, divided into approximately 4000 fractions, were employed during the isolation of the various pure compounds. Only a brief description is given of the details of isolation of the steroids identified.

**Pregnane-3α,17α,20α-triol**—When transfer of the non-ketonic extract (949 mg.) to the initial chromatogram was attempted, a large residue, insoluble in methylene chloride, was obtained. This product (228 mg.) was recrystallized from methanol and afforded 202 mg. of pure pregnane-3α, 17α,20α-triol, m.p. 249.5-252°; reported m.p. 252° (5), together with 20 mg. of less pure material judged to be principally pregnanetriol from the infrared spectrum. In addition, 170 mg. of pregnane-3α,17α,20α-triol, m.p. 249-251°, were obtained from the chromatogram. Thus a single compound represented over 40 per cent of the weight of the crude non-ketonic fraction.

**Δ18-Androstene-3α-ol**—This compound was the first steroid substance eluted from the chromatogram of the non-ketonic fraction. Trace amounts, insufficient for conventional isolation, were present; the infrared spectrum of the material was indistinguishable from the known compound (6). Two compounds, probably steroids but as yet unrecognized, were eluted after this product.

**Pregnane-3α,20α-diol**—Approximately 25 mg. of this compound were obtained from the chromatogram. Although the product was not completely pure, the infrared spectrum provided conclusive identification.

**Etiocholane-3α,17α-diol**—A small amount of this substance, identified by infrared spectrometry, was found in the chromatogram after pregnane-diol.

**11-Ketopregnane-3α,20α-diol**—6 mg. of this compound were crystallized from the eluates obtained after etiocholane-3α,17α-diol and identified by infrared spectrometry. The substance has been described in a previous report from these laboratories (7).

**Allopregnane-3α,17α,20α-triol**—Eluates containing this material (20 mg.) were obtained with 5 per cent ethanol in methylene chloride. A portion of this product was rechromatographed on paper in the system cyclohexane-toluene-propylene glycol to yield 4 mg. of product which after recrystallization from methanol melted at 228-230°, and its melting point was not depressed upon admixture with authentic allopregnane-3α,17α, 20α-triol, m.p. 227.5-230° (1). The infrared spectrum was indistinguishable from that of the reference substance.
Table I

Steroids Isolated from Urine of Patient with Congenital Adrenal Hyperplasia

<table>
<thead>
<tr>
<th>17-Ketosteroids</th>
<th>mg. per day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Androsterone</td>
<td>8.5</td>
</tr>
<tr>
<td>Etiocholanolone</td>
<td>10.2</td>
</tr>
<tr>
<td>Etiocholane-3,11,17-trione</td>
<td>Trace</td>
</tr>
<tr>
<td>3α-Hydroxyandrostane-11,17-dione</td>
<td>1.6</td>
</tr>
<tr>
<td>3α-Hydroxyetiocholane-11,17-dione</td>
<td>3.8</td>
</tr>
<tr>
<td>3α,11β-Dihydroxyandrostane-17-one</td>
<td>0.3</td>
</tr>
<tr>
<td>3α,11β-Dihydroxyetiocholane-17-one</td>
<td>1.7</td>
</tr>
<tr>
<td>Dehydroisoandrosterone</td>
<td>2.1</td>
</tr>
<tr>
<td>3β-Hydroxyetiocholane-17-one</td>
<td>1.4</td>
</tr>
<tr>
<td>3β-Hydroxy-Δ4-androstene-7,17-dione</td>
<td>0.8</td>
</tr>
<tr>
<td>3β-Hydroxyetiocholane-11,17-dione</td>
<td>Trace</td>
</tr>
<tr>
<td>11β-Hydroxyetiocholane-3,17-dione</td>
<td>“</td>
</tr>
</tbody>
</table>

20-Ketosteroids

| 3α-Hydroxyprogesterone-20-one | Trace |
| 3α,17α-Dihydroxyprogesterone-20-one | 12 |
| 3α,17α-Dihydroxyallopregnane-20-one | 1 |
| 3α,6α-Dihydroxyallopregnane-20-one | Trace |

Non-ketonic steroids

| Δ14-Androstene-3α-ol | Trace |
| Etiocholane-3α,17α-diol | “ |
| Pregnan-3α,20α-diol | 4 |
| 11-Ketopregnane-3α,20α-diol | 1 |
| Pregnan-3α,17α,20α-triol | 65 |
| Allopregnane-3α,17α,20α-triol | 3 |
| Allopregnane-3α,17α,20β-triol | Trace |
| 11-Ketopregnane-3α,17α,20α-triol | 12 |
| Pregnan-3α,11β,17α,20α-tetrol | 4 |

Quantitative values for the 17-ketosteroids were obtained by paper chromatography and application of the modified Zimmermann reaction (8, 9). The quantitative estimation of the other steroids is based on the amount isolated and is therefore a minimal value.

In addition to these compounds there were several artifacts found in the extract. These included traces of 3α-hydroxy-17α-pregnane-20-one and the n-homosteroids derived from 3α,17α-dihydroxyprogesterone-20-one. There were, as well, other steroids present as indicated from the infrared spectra of several fractions from the chromatogram.

Allopregnane-3α,17α,20β-triol—A small amount of this product was identified by infrared spectrometry in the paper chromatogram of the
previous compound; the amount present was insufficient for isolation in crystalline form.

11-Ketopregnane-3α,17α,20α-triol—Immediately after pregnane-3α,17α, 20α-triol, 75 mg. of almost pure 11-ketopregnane-3α,17α,20α-triol were eluted with 5 per cent ethanol in methylene chloride. The diacetate, obtained with acetic anhydride in pyridine and crystallized from acetone-methanol and from methanol, weighed 65 mg.; m.p. 225–227°; the infrared spectrum was identical with that of the reference compound (2); there was no depression of melting point upon admixture with the synthetic compound, m.p. 225–226°.

Pregnane-3α,11β,17α,20α-tetrol—Continued elution with 5 per cent ethanol in methylene chloride yielded 46 mg. of product containing this tetrol. Rechromatography on paper in the system chloroform-formamide gave 22 mg. of the free alcohol which was difficult to crystallize. Acetylation yielded pregnane-3α,11β,17α,20α-diol 3,20-diacetate, m.p. 209°, 215–218°. Upon admixture with the authentic sample, the m.p. was 212–218° (1); the infrared spectrum was identical with that of the synthetic sample.

**DISCUSSION**

It is known that a considerable number of non-ketonic steroids are present in human urine and that this steroid fraction can increase to relatively enormous amounts in patients with certain adrenal disorders. However, little precise information is available about the non-ketonic components, primarily because of inadequacy of methods for separation and quantitative determination of these polar steroids. The importance of non-ketonic metabolites has recently been emphasized by the observation that in man hydrocortisone is metabolized to an appreciable extent to yield completely saturated alcohols such as the cortols or compounds containing only a single unreactive carbonyl group, *e.g.* the cortolones (10). Since the existence of other related hydroxylated steroids was suspected, an examination of the non-ketonic components present in an extract of urine from a patient with congenital adrenal hyperplasia was undertaken. It was anticipated that with this disorder steroids normally present only in trace amount would be greatly increased in concentration. A better opportunity for identification would thus be afforded. In addition, it was felt that the unusual steroid pattern of the urine of the individual selected for the study was intrinsically interesting.

The study, while indicative of certain major pathways in the metabolism of steroid hormones, is perhaps more valuable for the information it may yield toward a proper approach to the study of the non-ketonic fraction. It is pertinent to note that the choice of a subject with congenital adrenal
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hyperplasia, while offering an opportunity to obtain isolable amounts of normally minor constituents, presented other technical difficulties in the isolation of steroids. Especially significant in this respect was the difficulty incurred by the relatively much larger amount of pregnane-3α,17α,20α-triol in the extract. This relatively insoluble component was distributed throughout a large number of fractions in the chromatogram and probably obscured small amounts of other products of comparable polarity. Equally, through its action as a displacement agent, it may have caused the elution of other compounds in unexpected positions in the chromatogram. Allied to these difficulties was the similar problem presented by the presence of relatively large amounts of 21-deoxysteroid which made difficult the identification of smaller amounts of related materials. For these reasons, our failure to isolate or identify certain expected components must be viewed with a certain reserve.

Comments on Congenital Adrenal Hyperplasia—The endocrine aspects of this disorder have received considerable attention (11–13). It is established that an inadequate synthesis of hydrocortisone is a fundamental defect in congenital adrenal hyperplasia. With this imperfection in mind it is important to emphasize the steroid pattern of the patient reported in this investigation and to compare that reported for the patient studied by Eberlein and Bongiovanni (11). Neither subject appeared capable of effecting all the stages of hydrocortisone biosynthesis. Among the twenty-five separate steroids found in the urine of our subject, there was no compound with a C-21 hydroxyl group. This negative finding, which should probably not be overemphasized, taken together with the demonstrated presence of large amounts of 21-carbon compounds oxygenated in all of the other positions characteristic of the hydrocortisone molecule, clearly indicates that one feature characteristic of this particular patient's adrenal abnormality was the relative inability to oxygenate C-21. There was, in addition, such a large amount of 11-oxygenated steroid isolated that there can be no question the adrenals of this patient were capable of more than adequate oxidation of C-11. These facts must be considered together with the evidence of enormous production of 11-deoxysteroid shown in Table I. Thus this patient exhibited a serious deficiency of 21-hydroxylation with an excessive amount both of steroids intermediate in hydrocortisone production and "adrenal androgen," defined by the metabolites androsterone and etiocholanolone (14). The patient of Eberlein and Bongiovanni was capable of a high level of C-21 hydroxylation as evidenced by the large quantity of metabolites of Reichstein's Substance S. Coupled with this, there was a complete absence of C-11-oxygenated steroids together with an androsterone and etiocholanolone output almost the same as our subject.
Therefore, two patients with the same fundamental disorder of inadequate hydrocortisone production each exhibited a different biochemical consequence of the imperfection. The one lacked the chemical mechanism to introduce oxygen at C-11; the other had a serious deficiency in oxygenation of C-21; otherwise both had all the necessary means to produce large amounts of steroids related to hydrocortisone. The excessive production of "adrenal androgen" was manifest in both subjects by the abundance of androsterone and etiocholanolone.

From these examples it can be concluded that the fundamental defect associated with congenital adrenal hyperplasia may be manifest by more than one chemical aberration. A consideration of these will be reported elsewhere. It should be emphasized, however, that the virilization associated with this condition is clearly reflected by the elevated production of androsterone and etiocholanolone. These two compounds are the principal steroids derived from the "adrenal androgen." By virtue of the increased adrenocorticotropic secretion resultant from deficiency of hydrocortisone, the normal elaboration of this adrenal component is elevated to a degree sufficient to produce the masculinization. Accordingly, the array of C-21 steroids is an expression of the relative inability of the gland to complete the synthesis of hydrocortisone, and the elevated androsterone and etiocholanolone excretions are a manifestation of the increased completed synthesis of the "adrenal androgen" precursor.

**SUMMARY**

1. The isolation of pregnane-3α, 11β, 17α, 20α-tetrol and allopregnane-3α, 17α, 20α-triol from the urine of a patient with congenital adrenal hyperplasia is described.

2. Evidence for the presence of allopregnane-3α, 17α, 20β-triol was obtained.

3. In addition, twenty-two previously described steroids were isolated and measured. The major "non-ketonic" urinary steroids were pregnane-3α, 17α, 20α-triol (65 mg. per day) and 11-ketopregnane-3α, 17α, 20α-triol (12 mg. per day).

4. Some comments are made on the chemical manifestations of the defect in steroid hormone synthesis associated with congenital adrenal hyperplasia.

The authors wish to express their gratitude to Dr. A. Kappas and Dr. E. A. Tsutsui for their cooperation with paper chromatography. We also wish to express our appreciation to the large group of devoted research assistants and technicians who made much of the work possible. The routine chemical and chromatographic separations were carried out by a
group under the supervision of Ruth Jandorek. The infrared spectrometry was under the supervision of Friederike Herling.

Addendum: We have recently had the opportunity to examine the urine of the patient by newer methods now available. It is quite clear that he is able to achieve a certain level of C-21 hydroxylation as shown by the isolation of cortol, cortolone, “tetrahydro E,” and “tetrahydro F” from his urine. The amount of these compounds present was at the lower limit of the normal range, by comparison with a small group of normal subjects. We take this opportunity to call attention to the limitation in C-21 hydroxylation, in contrast to a total absence of this reaction which might be misconstrued from consideration of the compounds isolated during the study described.

BIBLIOGRAPHY

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