CONCERNING THE CHARACTERIZATION OF POSSIBLE CORTICAL HORMONE METABOLITES IN URINE*

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Investigations of the nature of urinary steroids by both chemical (2, 3) and biological (4–7) methods have afforded certain tentative indications of the presence of substances having distinguishing characteristics associated with hormones of the adrenal cortex or of products of the metabolism of these hormones. Methods for either the determination or the selective fractionation of any such substances present in urinary extracts should advance the knowledge of intermediary hormone metabolism and might be of considerable clinical significance. The distinguishing oxygen function at C11 is present in some but not all of the recognized cortical steroids, and no known property associated with this function lends itself to either separation or analysis. A more general structural characteristic is the presence of the 3-carbon unit C17-C20-C21 in a uniquely high state of oxidation and comprising what may be described as a triose or desoxytriose unit. This paper describes orienting attempts to characterize possible cortical hormone metabolites in urine based upon the sugar-like nature of the side chain.

Oxidation with Periodic Acid

Among the known C21 cortical steroids, all those possessing characteristic biological activity, as well as certain of the inactive substances, have either the ketol grouping (a) or the 17-hydroxy ketol (dihydroxyacetone) grouping (b), while the glycerol grouping (c) is present in some of the inactive companion substances. All but two of the recognized C21 cortical steroids conform to one of the three patterns indicated. These structures are all susceptible to glycol cleavage with either periodic acid or lead tetraacetate. The ketols (a) are converted by these specific reagents into etio acids, the substances with the dihydroxyacetone grouping (b) are oxidized initially to α-hydroxy acids and then, with excess reagent, may yield 17-ketosteroids, and compounds possessing the glycerol side chain (c) afford 17-ketosteroids directly.

* For reviews and references see Reichstein and Reichstein and Shoppee (1).
Titration with Periodic Acid—The specificity of the glycol cleavage reaction, at least as applied to pure compounds, suggested the possibility that titration of urinary extracts with a suitable reagent might afford an index of the amount of cortical steroid metabolite present. The micro-method of Rappaport et al. (8) for the determination of certain hexoses with periodic acid was adapted to the problem of estimating water-insoluble steroids in pigmented urinary extracts by employing methanol as the solvent and determining the unutilized reagent by potentiometric titration with standard arsenious oxide solution. Trials with six 1 day non-ketonic steroid fractions of human urines were discouraging, for periodic acid was consumed in amounts far in excess of that which would be required if the entire fraction were composed of glycol components. Indeed it appeared that substances must be present that are capable of reducing periodic acid beyond the stage of iodic acid, the normal stopping point in the oxidation of glycols and glycerols. Williams and Woods (9) have shown that iodic acid is a powerful oxidizing agent for many different types of organic substances, even in the cold.
Oxidation of Non-Ketonic Steroid Fractions—Cortical steroid metabolites possessing the glycerol grouping (c) or the dihydroxyacetone grouping (b) should be convertible, by direct glycol cleavage or by cleavage and subsequent oxidation of the α-hydroxyetio acids, into 17-ketosteroids. The production of 17-ketosteroids from non-ketonic steroid fractions by oxidation with periodic acid or lead tetraacetate would thus constitute evidence of the presence of substances probably derived from the adrenal cortex. Since the tertiary hydroxyl group at C₁₇ is subject to ready elimination under the dehydrating action of mineral acids, acid-hydrolyzed urine undoubtedly is an unfavorable starting material for an investigation along the lines indicated. In the absence of material more favorable for study in quantity, experiments designed to determine whether glycol cleavage can give rise to the production of 17-ketosteroids were carried out with the non-ketonic neutral fraction derived from commercially processed acid-hydrolyzed pregnancy urine.

**Table I**

**Oxidation of Non-Ketonic Fraction of Pregnancy Urine**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Sample No.</th>
<th>Oxidant</th>
<th>Ketonic fraction produced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Weight</td>
</tr>
<tr>
<td>1</td>
<td>I. 50</td>
<td>HIO₄</td>
<td>1.07</td>
</tr>
<tr>
<td>2</td>
<td>Ia. 30</td>
<td>“</td>
<td>1.80</td>
</tr>
<tr>
<td>3</td>
<td>II. 30</td>
<td>“</td>
<td>0.70</td>
</tr>
<tr>
<td>4</td>
<td>“ 90</td>
<td>“</td>
<td>1.14</td>
</tr>
<tr>
<td>5</td>
<td>“ 30</td>
<td>Pb(OCOCH₃)₄</td>
<td>0.61</td>
</tr>
</tbody>
</table>

The results of five oxidation experiments are listed in Table I. Sample I, used in Experiment 1, had been reextracted with Girard's reagent to remove traces of ketones; a 50 gm. sample was oxidized with 5.0 gm. of crystalline periodic acid (dihydrate) in methanol (36 hours at 25°). This afforded 1.2 gm. of acidic material and a ketonic fraction amounting to 3.0 gm.; reextraction of the latter fraction with Girard's reagent gave 1.07 gm. of dark brown, glassy material having a value in the Callow-Zimmermann colorimetric determination for 17-ketosteroids of 140 mg. of androsterone equivalents. No precipitation was observed on treatment of portions with semicarbazide acetate and with digitonin, and no crystalline eluates were obtained when the material as such was chromatographed on alumina. Another portion (208 mg.) was separated with phthalic anhydride and the alcoholic fraction (59 mg.) and non-alcoholic fraction (107 mg.) were chromatographed on magnesium sulfate-Celite, but without success.

In Experiment 2, 100 gm. of Sample I were separated with phthalic
anhydride and the alcoholic fraction (Sample Ia, 30 gm.) was oxidized with periodic acid. The material extracted with Girard's reagent (1.8 gm.) was processed with phthalic anhydride and afforded 830 mg. of alcoholic ketonic steroids having a Callow value of 93 mg. (no precipitate with digitonin).

Sample II was obtained by processing the crude extract (615 gm.) from about 800 liters of human pregnancy urine as follows: The alcoholic fraction was segregated through the half phthalate and the material recovered (190 gm.) was treated three times with Girard's reagent to remove all traces of ketones from the residual neutral non-ketonic alcoholic fraction (154 gm.). Three large scale glycol oxidation reactions were conducted and ketonic fractions were obtained in 2 to 4 per cent yield (Table I), but no crystalline products could be isolated. In Experiment 4 the reaction mixture afforded 1.46 gm. of acidic material and 4.14 gm. of an oily ketonic fraction, and the latter had a colorimetric value of 870 mg. of androsterone equivalents. Seven chromatograms of the ketonic fraction, involving a total of 421 eluates, failed to afford crystalline products.

Ketosteroid Assay Following Periodic Acid Oxidation—Several ketonic fractions from acid-hydrolyzed human urines were oxidized with periodic acid in methanol, but no increase in 17-ketosteroid content was noted and in several instances there was a distinct drop in the Callow value. On the other hand, the oxidation of synthetic Δ⁴-pregnenetriol-17α,20,21-one-3 (10 mg.) with 2 parts of periodic acid dihydrate resulted in an increase in Callow value from 4.1 to 14.1 mg. (theoretical increase, 8.2 mg.). Such other early experiments in the direction of the development of an assay method based upon the 17-ketosteroid content after glycol cleavage were of a preliminary nature and are superceded by the more extensive study reported in a paper by Talbot and Eitingon (10), with whom we have exchanged information.

Acetal Formation

A second possible method investigated for the characterization of cortical steroid metabolites having a highly hydroxylated side chain was by condensation with an aldehyde so substituted that the expected acetal derivatives of any 1,2- or 1,3-glycols present could be selectively extracted from a mixture with acid or with base. The steroids of types (b) to (c), listed above, should all afford cyclic acetals, and by analogy to known compounds these should be stable to alkali and possibly subject to cleavage to the original steroids under mild conditions of acid hydrolysis.

In exploratory experiments several acetals were prepared by the condensation of simple model glycols with variously substituted benzenaldehydes. The technique introduced by Salmi (11) for the preparation of otherwise difficultly accessible ketals proved advantageous, and the best results were
obtained by using benzene or ethyl acetate as solvent and slowly distilling
the mixture of components, solvent, and catalyst to remove the water
formed in the equilibrium reaction. A trace of p-toluenesulfonic acid
effectively catalyzed the reactions, but all attempts to find non-acidic
catalysts which would be more sparing of the sensitive C11- and C17-hydroxyl
groups of the cortical steroids were unsuccessful. Thus calcium chloride,
ferric chloride, ammonium chloride, and ammonium sulfate (12) all proved
unsatisfactory. The model glycols investigated were ethylene glycol,
1,2-propylene glycol, and 1-hydroxymethylcyclohexanol-1 (I), and the
aldehydic components were m- and p-nitrobenzaldehyde, terephthalaldehyde
methyl ester, o-, m-, and p-hydroxybenzaldehyde. Thus a typical
example is the preparation of the acetal II.

Typical Procedure—In a representative preparation a solution of 2.0 gm.
of p-nitrobenzaldehyde, 2.0 cc. of redistilled ethylene glycol, and a few
crystals of p-toluenesulfonic acid in 300 cc. of dry benzene was refluxed
under a special take-off condenser (13) of the type used by Salmi (11) at
such a rate that at the end of 2 hours about three-fourths of the solvent had
been distilled. 200 cc. of ethyl acetate were added and the solution was
washed with water, dried over sodium sulfate, and evaporated in vacuo.
The residue on cooling was a white crystalline solid, m.p. 91-94°, consisting
of the very nearly pure acetal (it depressed the melting point of p-nitro-
benzaldehyde). The analytical sample was recrystallized from acetone
and melted at 89-89.5°. The acetal was hydrolyzed readily by hot dilute
acetic acid and afforded p-nitrobenzaldehyde. Equally satisfactory results
were obtained with the use of ethyl acetate as solvent and with potassium
acid sulfate as the catalyst, but with the neutral agents cited above no
acetal formation was noted.

Properties and Analyses of Model Acetals—The acetals listed in Table II
were prepared by the procedure outlined, with the yields noted.
The nitrobenzaldehydes afford glycol acetals in good yield, but there
seems to be little promise of developing a procedure of separation based
### Table II

**Model Acetals**

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<tr>
<th>Formula</th>
<th>Components</th>
<th>Yield</th>
<th>C</th>
<th>H</th>
<th>C</th>
<th>H</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Calculated</td>
<td>Found</td>
<td>Calculated</td>
<td>Found</td>
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<tr>
<td>CH₃O⁻</td>
<td>HOCH₂CH₂OH,</td>
<td>98.5</td>
<td>55.38</td>
<td>55.41</td>
<td>4.65</td>
<td>4.73</td>
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<tr>
<td>CH₃O⁻</td>
<td>p-NO₂C₆H₄CHO</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>CH₃CHO⁻</td>
<td>HOCH₂CH₂OH,</td>
<td>80</td>
<td>57.38</td>
<td>57.73</td>
<td>5.30</td>
<td>5.19</td>
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<tr>
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<tr>
<td>CH₃CHO⁻</td>
<td>HOCH₂CH₂OH,</td>
<td>70</td>
<td>55.40</td>
<td>55.62</td>
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<tr>
<td>CH₃O⁻</td>
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<tr>
<td>CH₃CHO⁻</td>
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<td>57.44</td>
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<tr>
<td>O⁻-CH₂C₆H₄NO₂⁻</td>
<td>I, p-NO₂C₆H₄CHO</td>
<td>65</td>
<td>63.81</td>
<td>63.99</td>
<td>6.51</td>
<td>6.29</td>
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<tr>
<td>CH₃O⁻</td>
<td>I, m-NO₂C₆H₄CHO</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>O⁻-CH₂C₆H₄NO₂⁻</td>
<td>I, m-NO₂C₆H₄CHO</td>
<td>55</td>
<td>63.81</td>
<td>64.04</td>
<td>6.51</td>
<td>6.32</td>
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</table>
upon reduction and extraction of the amine derivative with acid, for this probably would result in cleavage of the acetal linkage. The free phthalaldehydeic acids appeared unpromising as reagents because of their sparing solubility in non-aqueous solvents and because of the opportunity for ester formation by interaction with steroid hydroxyl groups. Terephthalaldehyde methyl ester could be condensed satisfactorily with model glycols, but such a reagent would have the disadvantage of affording opportunity for the damaging of alkali-sensitive steroids in the saponification of the esterified product prior to extraction. The phenolic reagents are free from the limitations enumerated, and m-hydroxybenzaldehyde gave better yields than the isomers and thus proved to be the most satisfactory aldehydeic reagent for the purpose at hand.

**Acetal Separation of a Model Glycol**—As a means of evaluating the scheme of separation an attempt was made to isolate 1-hydroxymethylcyclohexanol-1 from a mixture of the glycol (10.3 gm.) with mineral oil (50 cc.). A solution of the mixture in 800 cc. of benzene containing 30 mg. of p-toluenesulfonic acid and 10 gm. of m-hydroxybenzaldehyde was refluxed for 2 hours, during which time 400 cc. of distillate were allowed to collect. The residual solution was extracted with 2 N sodium hydroxide and the alkaline extract was cooled to 0°, neutralized carefully with dilute acetic acid solution, and extracted with ether. Distillation of the material recovered from the washed and dried ethereal extract afforded 12.4 gm. (66 per cent) of the acetal as a viscous oil (b.p. 174° at 1 mm.) that crystallized on standing (m.p. 73-74°). For hydrolysis, a solution of 7.3 gm. of the acetal in 30 cc. of 50 per cent acetic acid was heated for 3 hours on the steam bath. The solvent was removed in vacuo and the residue dissolved in ether and extracted with 2 N sodium hydroxide. The neutral fraction on recrystallization from ether gave 1.3 gm. of 1-hydroxymethylcyclohexanol-1, representing a 22 per cent over-all recovery.

**Acetal Separation of Model Steroids**—One of two compounds of the steroid series investigated as models of cortical hormone metabolites was
Δ^4-pregnenetriol-17α,20,21-one-3 (III). This was prepared by known reactions, starting with the Oppenauer oxidation of Δ^5-17-ethynyl-\text{-}androstenediol\textsuperscript{1} to Δ^4-17-ethynyl-\text{-}androstenol-17-one-3 ((14), yield 66 per cent). Reduction of the acetylenic substance in pyridine with Raney’s nickel or with 2 per cent palladium on calcium carbonate (15) afforded Δ^4-17-vinyl-\text{-}androstenol-17-one-3 in 70 per cent yield; the hydrogenation proceeded smoothly at room temperature and pressure and came to an abrupt stop at the desired stage. The triol III was obtained in 40 per cent yield by hydroxylation with osmium tetroxide (16) and reductive hydrolysis of the osmic ester with sodium sulfite; the purified substance melted at 234.5–235.5°, corrected. The volatile osmium tetroxide was conveniently stored in the form of a standard solution in carbon tetrachloride and kept in a flask with a tightly fitting ground glass stopper.

On treatment of the triol III in benzene solution with \textit{m}-hydroxybenzaldehyde and a trace of \textit{p}-toluenesulfonic acid, 75 per cent of the substance was converted into alkali-soluble material, presumably the acetal. The neutral fraction was not the unchanged triol but an uncrystallizable oil, probably resulting from the dehydration of III. The alkaline extract was cooled to 0° and carefully acidified with dilute acetic acid and extracted with ether. The crude acetal recovered from the ethereal extract was submitted to very mild hydrolysis with dilute acetic acid but the resulting neutral fraction was an oil which failed to crystallize. Experiments with the pure triol III then indicated that the difficulty probably is in the very great sensitivity of the substance to the dehydrating action of acids. Re-fluxing the triol in benzene solution with a trace of \textit{p}-toluenesulfonic acid for 2 hours resulted in the conversion of 40 per cent of the material into a resinous product. Treatment with dilute acetic acid or with anhydrous copper sulfate also resulted in considerable alteration. Reichstein and coworkers (17, 18), working with compounds similar to III but of the \textit{C}17-configuration, were able to effect satisfactory conversion to the acetonide derivatives with the use of anhydrous copper sulfate as catalyst, but this case is different from the present one because a large excess of the carbonyl compound (acetone) could be employed.

The second steroid studied was Δ^4-pregnenetriol-3β,20,21 (IV), prepared by aluminum isopropoxide reduction of Δ^4-3-hydroxy-21-acetoxy-pregnenenone-20\textsuperscript{1} and hydrolysis, according to Steiger and Reichstein (18). This substance has no tertiary hydroxyl group at C17 and should be less sensitive to dehydration than III. A solution of 100 mg. of IV (stereoisomeric mixture, m.p. 201–210°), 100 mg. of \textit{m}-hydroxybenzaldehyde, and 20 mg. of \textit{p}-toluenesulfonic acid in 1.2 liters of dry benzene was refluxed

\textsuperscript{1} For supplies of this material we are greatly indebted to Dr. Erwin Schwenk, the Schering Corporation, and to Dr. C. R. Scholz, the Ciba Pharmaceutical Products, Inc.
for 18 hours, during which time about half of the solvent was allowed to distil. Partition with alkali afforded an oily neutral fraction amounting to 60 mg.; the alkali-soluble acetal fraction was acidified with excess hydrochloric acid to effect hydrolysis, and the resulting neutral material yielded, after recrystallization, 20 mg. of the original $\Delta^2$-pregnenetriol-3(\(\beta\)), 20, 21 (IV), m.p. 205–215°.

These orienting experiments are not very encouraging. The triol III, with a tertiary a-hydroxyl at C17, is subject to alteration (probably dehydration) under the conditions required for the formation and hydrolysis of the acetal derivative. In the case of the triol IV, which has no tertiary hydroxyl groups, conversion to the alkali-extractable acetal of m-hydroxybenzaldehyde and regeneration of the original steroid can be realized, but the yields in both steps are far from quantitative. The acetal separation procedure was tried on a sample of extract of the acid-hydrolyzed pregnancy urine described above, but a twice processed "glycol fraction" afforded no crystalline products.

We are greatly indebted to Dr. C. P. Rhoads and Dr. K. Dobriner, of Memorial Hospital, New York, for supplies of hormone fractions, for colorimetric determinations, and for their general cooperation. This project was conducted in part under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Memorial Hospital.

**SUMMARY**

Two possible methods are suggested for characterizing cortical hormone metabolites that may appear in the urine. Both methods are based on the sugar-like nature of the highly oxygenated side chain. One, involving glycol cleavage with periodic acid or lead tetraacetate, offers some promise. The other, involving conversion of a 1,2- or 1,3-glycol to an alkali-extractable acetal derivative, is subject to the serious limitation that the conditions of acid catalysis required for the formation and cleavage of the acetals are sufficiently severe to cause some elimination of the acid-sensitive C11- and C17-hydroxyl groups.

**BIBLIOGRAPHY**

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