HYDROLYSIS OF URINARY CONJUGATED 17-KETOSTEROIDS BY ACETATE BUFFER

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It has long been recognized that the usual methods for cleavage of steroidal conjugates by strong acids may effect various transformations (1). In an earlier publication (1) one particular alteration, the conversion of a 3β-hydroxy-17-ketosteroid to a non-digitonin-precipitable ("α")-17-ketosteroid1 by standard HCl hydrolysis was studied. This conversion could be avoided by use of the hydrolytic procedure devised for sodium dehydroisoandrosterone sulfate in water or urine by Talbot et al. (2) with barium chloride in acetate buffer, pH 5.8. The present paper reports a series of experiments designed to determine (a) the essential factors involved in this hydrolytic procedure and (b) the nature of the conjugated 17-ketosteroids of normal male urine which are hydrolyzed by this treatment.

Methods

Colorimetric Determination of 17-Ketosteroids—The 17-ketosteroid content of the urine extracts was measured by the Zimmermann reaction essentially as modified by Holtorff and Koch (3).

Preparation of Urine2 Extracts for Color Assay—After hydrolysis, the free 17-ketosteroids were extracted from the urine by the procedure of Talbot and Eitingon (4), CC4 being used as the extracting agent instead of ethyl acetate. The ketonic fraction was removed by the Pincus and Pearlman modification (5) of the Girard method and then separated into "α" and β fractions by the digitonin precipitation method of Frame (6).

Hydrolysis of Conjugated 17-Ketosteroids. With Hydrochloric Acid—The

* Aided by grants from the Graduate School of the University of Minnesota and the United States Public Health Service.
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1 This term is used to designate non-digitonin-precipitable compounds; namely, those with a C17 hydroxyl trans to the C19 methyl, or compounds without a C17 hydroxyl group.
2 All the urine specimens used in this study were taken from batches of pooled male urine.
procedure of Talbot et al. (7) was followed in which concentrated HCl is added to the urine in the proportion of 15 per cent by volume, and the mixture boiled under a reflux for 10 minutes. Since no method of hydrolysis has yet been reported which yields a greater assayable amount of ketosteroids than such an acid hydrolysis, the ketosteroids released by acid are referred to in this paper as the "total" urinary ketosteroids.

With Barium Chloride—The technique of Talbot et al. (2, 4) was followed in which the residue from an alkali-washed butyl alcohol extract of neutral urine is heated in acetate buffer (pH 5.8) containing barium chloride to liberate the 17-ketosteroids.

EXPERIMENTAL

Acetate Buffer Hydrolytic Procedure—No supporting evidence for the effectiveness of the various procedures employed in the Talbot hydrolytic technique is given (2). Therefore it was felt that such studies should be carried out and only the most efficient procedures used to determine the nature of the conjugates hydrolyzed.

Effect of pH on Extraction by Butanol of Conjugated 17-Ketosteroids from Urine—Butanol concentrates of the conjugated 17-ketosteroids have been prepared by other workers at both neutral pH (2, 4, 8) and at pH 1.0 (9). The effect of pH on the distribution of the conjugated 17-ketosteroids between urine and butanol was therefore first investigated.

Samples of 800 cc. of urine (Batch C) were adjusted to pH 1, 3, 5, 7, 9, and 11, respectively, each was extracted four times with $\frac{3}{4}$ volume of butanol, and the butanol residues were assayed for ketosteroid content after HCl and after the buffer-BaCl$_2$ hydrolytic procedures. The buffer-BaCl$_2$ ketosteroid fractions obtained for the pH 3, 7, and 11 BuOH residues were further fractionated with digitonin. The results are shown in Table I (800 cc. of urine, Batch C, assayed for 8.1 mg. of "total" 17-ketosteroids).

It is readily seen that, while a practically complete removal of the conjugated 17-ketosteroids from urine by butanol is effected at pH 1 or 3, considerable amounts of these ketosteroids are left in the urine extracted at pH above 3. In view of these observations, it was not surprising to find that washing the butanol extracts with aqueous alkali resulted in additional losses of conjugated 17-ketosteroids. It is further seen from the data shown in Table I that the buffer-BaCl$_2$-hydrolyzable conjugates were removed from urine by butanol at any pH. However, it was felt that an increased measure of safety in the preparation of the butanol extracts would be attained by effecting the extractions and washings at pH less than 3.0 (Diagram 1). The extracts thus obtained were sufficiently free of urea to eliminate the hazard of NH$_3$ formation on pH during the subsequent hydrolysis (2).
<table>
<thead>
<tr>
<th>pH of urine, BuOH-extracted</th>
<th>17-Ketosteroids in BuOH extract</th>
<th>Hydrolyzed by HCl</th>
<th>Hydrolyzed by buffer-BaCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg.</td>
<td>mg.</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>7.8</td>
<td>1.5</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>7.6</td>
<td>1.7</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>6.3</td>
<td>0.6</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>5.4</td>
<td>0.7</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>5.1</td>
<td>0.9</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>4.3</td>
<td>0.9</td>
</tr>
</tbody>
</table>

### Table I

**Effect of pH on Extraction of Ketosteroid Conjugates from Urine by Butanol**

<table>
<thead>
<tr>
<th>pH of urine, BuOH-extracted</th>
<th>17-Ketosteroids in BuOH extract</th>
<th>Hydrolyzed by HCl</th>
<th>Hydrolyzed by buffer-BaCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg.</td>
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<td>1</td>
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<td>3</td>
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<tr>
<td>5</td>
<td></td>
<td>6.3</td>
<td>0.6</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>5.4</td>
<td>0.7</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>5.1</td>
<td>0.9</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>4.3</td>
<td>0.9</td>
</tr>
</tbody>
</table>

### Diagram 1

**Buffer Hydrolysis of Conjugated Urinary 17-Ketosteroids**

Urine (400-1000 cc.)

To pH 1.0 with concentrated HCl; extract 4 times with 1/4 volume n-BuOH

BuOH Urine (discard)

Wash 6 times with 1/16 volume 0.1 N HCl

0.1 N HCl (discard) BuOH

+ NaOH to pH 7; distill to dryness in vacuo at bath temperature not exceeding 50°

BuOH residue

Dissolve in 100 cc. 0.1 N acetate buffer, pH 4.7

Heat 4 hrs. in boiling water bath; extract with CCl₄, etc.

Buffer-hydrolyzable 17-ketosteroids
Role of Barium Chloride in Buffer Hydrolysis of Conjugated 17-Ketosteroids—Barium chloride in a concentration of 15 gm. per cent in the acetate buffer medium (pH 5.8) was recommended (2) on the theory that it would facilitate hydrolysis by removing sulfate ions from solution through the formation of insoluble barium sulfate. Since no experimental data were supplied in support of this hypothesis, the value of the BaCl₂ in the hydrolytic procedure was therefore investigated. The residues obtained from the butanol extracts of normal male urine (each aliquot was equivalent to 400 cc. of urine) were prepared according to the procedure outlined above, dissolved in 100 cc. of 0.1 N HAc-NaAc buffer (pH 5.8) and, after the addition of 15 gm. per cent of BaCl₂ to some of the samples, heated in a boiling water bath for 4 hours. The liberated 17-ketosteroids were then determined. The results are shown in Table II.

It is readily seen that the same degree of hydrolysis occurred in all cases in the samples without BaCl₂ as in those with BaCl₂, and that similar amounts of β-17-ketosteroids were liberated in each case.

Effect of pH on Hydrolysis of Conjugated 17-Ketosteroids—Aliquots of the residues obtained from the BuOH extracts of two batches of urine were dissolved in 0.1 N acetate buffer at different acid pH values (pH below 3 was attained by the addition of HCl to the acetate buffer). After heating in a boiling water bath for 4 hours, the liberated 17-ketosteroids were determined. The results are graphically shown in Fig. 1.

It is seen that the pH at which the butanol extracts are heated has little effect on the amount of 17-ketosteroids liberated until a pH of 3 or less has been achieved. The amount of conjugated 17-ketosteroids liberated in the pH range of 4 to 6 under these conditions is approximately constant and is about 25 per cent of the total present in the urine. 0.1 N acetate

### Table II

<table>
<thead>
<tr>
<th>Urine batch</th>
<th>BaCl₂ present</th>
<th>17-Ketosteroids liberated</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>+</td>
<td>Before fractionation (mg.)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.8</td>
</tr>
<tr>
<td>“</td>
<td></td>
<td>1.7</td>
</tr>
<tr>
<td>A</td>
<td>+</td>
<td>1.3</td>
</tr>
<tr>
<td>“</td>
<td></td>
<td>1.3</td>
</tr>
<tr>
<td>H</td>
<td>+</td>
<td>1.1</td>
</tr>
<tr>
<td>“</td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>J</td>
<td>+</td>
<td>1.7</td>
</tr>
<tr>
<td>“</td>
<td></td>
<td>1.9</td>
</tr>
</tbody>
</table>
buffer, pH 4.7, was adopted as the hydrolytic medium in subsequent procedures, since the buffer pair of acetic acid-sodium acetate has the greatest buffering capacity at this pH and affords a better buffering medium than does a buffer of pH 5.8 for any NH₃ released during the hydrolysis.

**Fig. 1.** Effect of pH on the hydrolysis of conjugated 17-ketosteroids in urine

**Fig. 2.** Effect of heating time on buffer hydrolysis of conjugated 17-ketosteroids in urine.

**Effect of Time of Heating in Acetate Buffer on Hydrolysis of Conjugated 17-Ketosteroids**—The residues of butanol extracts of normal male urine (Batch G) were dissolved in 0.1 N acetate buffer (pH 5.8) and heated on a boiling water bath for periods of 1, 2, 4, 8, and 24 hours respectively. The amounts of 17-ketosteroids liberated under these conditions are graphically shown in Fig. 2. The hydrolysis was essentially complete (85 per cent) at 4 hours. The fraction of ketosteroids not liberated by buffer was recoverable in all cases by subsequent hydrolysis with HCl, indicating the
non-destructiveness of prolonged heating in buffer on the liberated ketosteroids (see also the hydrolysis of dehydroisoandrosterone sulfate, Table IV).

**Routine Buffer Hydrolytic Procedure**—On the basis of the findings reported above, a procedure was developed for the hydrolysis of the conjugated 17-ketosteroids of normal male urine by means of acetate buffer. It is shown schematically in Diagram 1.

**Nature of Conjugated 17-Ketosteroids Hydrolyzed by Acetate Buffer Technique.** *Total Amounts of 17-Ketosteroids Released by Buffer Hydrolysis*—During the course of the investigations reported in this paper, samples from eighteen batches of pooled male urine have been hydrolyzed with HCl, and seventeen by the buffer technique. The ketosteroid fractions of eleven of these batches were further fractionated with digitonin. (Most of the experiments were carried out at least in duplicate.)

The buffer hydrolysis released about one-fourth (15.2 to 34.5 per cent, average 23.1 per cent) of the "total" 17-ketosteroids in urine. Approximately 60 per cent of the compounds released were of the $\beta$ configuration (or 14 per cent (12.3 to 22.6 per cent) of the "total" 17-ketosteroids in the urine). Following HCl treatment, an average of only 4.6 per cent (1.2 to 8.9 per cent) could be determined as $\beta$ compounds. This latter figure is in close agreement with that reported by other authors (1).

**Relationship of Buffer and Glucuronidase-Hydrolyzed Ketosteroid Conjugates**—Glucuronidase preparations of both animal (2, 10, 11) and bacterial (12, 13) origin have been employed for the hydrolysis of steroid glucuronides in urine. A method in which a calf spleen glucuronidase concentrate is employed for such hydrolyses has been developed in our laboratory* and was applied to butanol concentrates of normal male urine specimens. Approximately 40 per cent of the "total" 17-ketosteroid content of normal male urine was liberated by this enzymatic hydrolysis, the $\beta$ fraction being equivalent to 2.8 per cent of the "total" 17-ketosteroids. This small amount of the $\beta$ compounds in the 17-ketosteroids liberated by glucuronidase suggested that the group of conjugated steroids hydrolyzed by buffer was not being attacked by the glucuronidase treatment. This was experimentally tested by carrying out buffer and enzyme hydrolyses in sequence on butanol residue concentrates of the same urine sample (urine Batch B2) (Table III). Confirmatory data were obtained from experiments with two other batches of urine (A2 and C2).

The results of this experiment supported the thesis that different groups of conjugates were being attacked by the buffer and enzyme treatments. The amount of 17-ketosteroids liberated by buffer was not affected by

previous enzyme hydrolysis (Experiment 3), thereby demonstrating that the glucuronidase treatment did not attack the conjugates hydrolyzed by the buffer treatment. Conversely, the buffer treatment did not hydrolyze ketosteroid glucuronides, since previous buffer treatment (Experiment 4) did not reduce the amount of 17-ketosteroids which could be obtained by glucuronidase hydrolysis.

Hydrolysis of Pure Steroid Sulfates by Buffer Technique—The hypothesis that 17-ketosteroid sulfates may be hydrolyzed by the buffer treatment was tested by subjecting a number of pure steroid sulfates to the conditions of the buffer hydrolysis. The results of the buffer hydrolysis on the steroid sulfates studied are shown in Table IV. A few mg. (1.0 to 5.5

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Hydrolytic procedure</th>
<th>17-Ketosteroids released</th>
<th>Total</th>
<th>&quot;α&quot;</th>
<th>β</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HCl</td>
<td>5.5</td>
<td>mg.</td>
<td>4.8</td>
<td>0.2</td>
</tr>
<tr>
<td>2</td>
<td>Buffer</td>
<td>1.4</td>
<td>mg.</td>
<td>0.4</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>HCl</td>
<td>3.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Enzyme</td>
<td>2.4</td>
<td>mg.</td>
<td>2.0</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Buffer</td>
<td>1.3</td>
<td>mg.</td>
<td>0.4</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>HCl</td>
<td>2.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Buffer</td>
<td>1.5</td>
<td>mg.</td>
<td>0.4</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>Enzyme</td>
<td>3.0</td>
<td>mg.</td>
<td>2.8</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>HCl</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

mg.) of the steroid sulfate were dissolved in 100 cc. of 0.1 N acetate buffer, pH 4.7, heated on a boiling water bath for varying periods of time, and the liberated steroids extracted with CCl₄ or ether. The free steroids in the extracts were assayed in the following manner: androsterone, isoan-

4 The sodium dehydroisoandrosterone sulfate, sodium androsterone sulfate, and sodium testosterone sulfate were obtained through the courtesy of Dr. C. R. Scholz, Ciba Pharmaceutical Products, Inc. Sodium estrone sulfate was kindly supplied by Dr. S. Cook of Ayerst, McKenna and Harrison, Ltd. Sodium estradiol sulfate was obtained through the courtesy of Dr. G. W. Holden of Charles E. Frosst and Company. Sodium epicholesterol sulfate was obtained through the courtesy of Dr. S. Lieberman of the Sloan-Kettering Institute for Cancer Research, who also supplied a small batch of sodium cholesterol sulfate. A second batch of sodium cholesterol sulfate was prepared by A. Zuckerbraun of the Department of Chemistry of the University of Minnesota. Sodium isoandrosterone sulfate was also prepared by Mr. Zuckerbraun from isoandrosterone acetate kindly supplied by Dr. C. R. Scholz of the Ciba Pharmaceutical Products, Inc.
drosterone, testosterone, and dehydroisoandrosterone were assayed by the routine Zimmermann reaction with the corresponding steroid as the standard; estradiol and estrone were determined by the method of Stevenson and Marrian (16); cholesterol was assayed by the modified Liebermann-Burchard reaction of Schoenheimer and Sperry (17); epicholestanol was determined gravimetrically.

It is seen that those compounds containing both a \( \beta \) configuration of the sulfate and a \( \beta-\gamma \) unsaturation to this grouping (sodium dehydroisoandrosterone sulfate and sodium cholesterol sulfate) are hydrolyzed by the buffer hydrolysis.

### Table IV

<table>
<thead>
<tr>
<th>Sodium sulfate of</th>
<th>Configuration of (-\text{OH} (-\text{OSO}_3\text{H}))</th>
<th>Presence of (\beta-\gamma) unsaturation</th>
<th>Per cent hydrolysis by buffer after</th>
</tr>
</thead>
</table>
| Androsterone.. . . | \(\alpha\) | - | 7 | 4
| Epicholestanol. . . | " | - | 2 | 3
| Isoandrosterone. . . | \(\beta\) | - | 5 | 7
| Testosterone* | " | - | 2 | 2
| Estradiol* | " | - | 1 | 2
| Estrone. . . . | Phenolic | - | 0 | 0
| Dehydroisoandrosterone | \(\beta\) | + | 65 | 95 | 98
| Cholesterol. . . | " | + | 45 | 64 | 95

* Holden and Bromley have reported on the inability of the BaCl₂-buffer (pH 5.8) technique (2) to hydrolyze estradiol sulfate (14) and testosterone sulfate (15).

**Isolation and Characterization of 17-Ketosteroids Released from Urine by Buffer Hydrolysis; Separation of Buffer-Hydrolyzable 17-Ketosteroids**—100 liters of normal male urine were extracted within 24 hours after collection in 10 to 20 liter batches as collected. Concentrated HCl was added to bring the pH to 1.0 and a butanol concentrate prepared according to the routine procedure (see Diagram 1). The “\(\alpha\)”- and \(\beta\)-hydroxy ketosteroid fractions were obtained by procedures schematically shown in Diagram 2.

**Fractionation of \(\beta\)-Hydroxy Ketosteroids Released by Buffer Hydrolysis; Isolation and Identification of Dehydroisoandrosterone**—The \(\beta\)-hydroxy ketosteroid fraction (Diagram 2) was relatively pure (110 mg. of 17-ketosteroid in a residue weight of 152.5 mg.; i.e., 72 per cent ketosteroid) and direct crystallization from ethanol-water was therefore attempted. Two batches of crystals were obtained (total weight, 48.5 mg.) which melted at approx-

* All melting points were determined on a Fisher micro melting point apparatus and are uncorrected.
approximately 132°. Smaller amounts of other crystalline material melted below this point and were therefore combined with the mother liquors, placed on a 15 gm. aluminum oxide column (aluminum adsorption, Fisher Scientific Company), and eluted with 100 to 500 cc. portions of pentane-benzene, benzene-chloroform, chloroform, and methanol in order. The resi-

**Diagram 2**

*Separation of Buffer-Hydrolyzable 17-Ketosteroids from Normal Male Urine*

Residue from BuOH extract of conjugated 17-ketosteroids from 100 liters urine

- In 2 liters 0.1 N acetate buffer, pH 4.7, at 100° 4 hrs.; cooled, extracted with CCl₄
- CCl₄: buffer-hydrolyzable 17-ketosteroids (weight, 2.5 gm.)
- Aqueous non-buffer-hydrolyzable 17-ketosteroids
- Girard's reagent T
- Non-ketonic fraction (weight, 1.54 gm.)
- Ketonic fraction (weight, 397 mg., 17-ketosteroids, 220 mg.)
- Digitonin
- Digitonin-non-precipitable ("α"-ketosteroids) (weight, 213 mg.; 17-ketosteroids, 86 mg.)
- Digitonin-precipitable (β-ketosteroids) (weight, 152.5 mg.; 17-ketosteroids, 110 mg.)

Residues from the 1:4 CHCl₃-benzene eluates were semicrystalline (combined residue weight, 53 mg.; assayable 17-ketosteroids, 47 mg.), and on crystallization from EtOH-H₂O yielded 30.7 mg. of crystals (m.p. about 134°). A total of 79.2 mg. of apparently similar crystalline material was thus isolated from the β fraction of the buffer-hydrolyzable 17-ketosteroids of 100 liters of pooled normal male urine. On recrystallization the crystals melted at 137–139° and, when mixed with a sample of pure dehydroisoandrosterone (m.p. 137–139°), showed no melting point depression.
After drying at 70° for 10 hours at 0.05 mm. of Hg pressure, a sample was analyzed for carbon and hydrogen.

\[ \text{C}_{18}\text{H}_{20}\text{O}_2 \]. Calculated, C 79.12, H 9.78; found, C 78.61, H 9.99

\([\alpha]_D^2 = +11.4^\circ \) (ethanol); dehydroisoandrosterone \([\alpha]_D = +10.9^\circ \) (ethanol) (18)

The acetate was prepared in a pyridine-acetic anhydride mixture and, after recrystallization from ethanol-water, melted at 165–167°. No mixed

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Eluent</th>
<th>Volume of eluent</th>
<th>17-Ketosteroids recovered</th>
<th>Weight</th>
<th>M.P.</th>
<th>Subsequent treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pentane-benzene 1:1</td>
<td>300 cc.</td>
<td>8.4 mg.</td>
<td>27.3 mg.</td>
<td>Oily</td>
<td>Combined with mother liquor of Fraction 2 and chromatographed</td>
</tr>
<tr>
<td>2</td>
<td>Pentane-benzene 1:1</td>
<td>60 cc.</td>
<td>17.8 mg.</td>
<td>15.4 mg.</td>
<td>139–144</td>
<td>Crystallization; 5.8 mg. crystals obtained (156–158°)</td>
</tr>
<tr>
<td>3</td>
<td>Benzene</td>
<td>300 cc.</td>
<td>15.5 mg.</td>
<td>27.6 mg.</td>
<td>127–134</td>
<td>Chromatographed; no crystalline material obtained</td>
</tr>
<tr>
<td>4</td>
<td>&quot;</td>
<td>100 cc.</td>
<td>2.2 mg.</td>
<td>16.5 mg.</td>
<td>Oily</td>
<td>Discard</td>
</tr>
<tr>
<td>5</td>
<td>&quot;</td>
<td>100 cc.</td>
<td>7.9 mg.</td>
<td>12.1 mg.</td>
<td>130–146</td>
<td>Girard reaction; no crystalline material obtained</td>
</tr>
<tr>
<td>6</td>
<td>&quot;</td>
<td>900 cc.</td>
<td>11.2 mg.</td>
<td>29.8 mg.</td>
<td>Oily</td>
<td>As for Fraction 5</td>
</tr>
<tr>
<td>7</td>
<td>&quot;</td>
<td>1000 cc.</td>
<td>3.8 mg.</td>
<td>13.1 mg.</td>
<td>&quot;</td>
<td>Discard</td>
</tr>
<tr>
<td>8</td>
<td>Chloroform-benzene 1:3</td>
<td>500 cc.</td>
<td>2.7 mg.</td>
<td>11.0 mg.</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>9</td>
<td>Chloroform-benzene 1:1</td>
<td>700 cc.</td>
<td>22.6 mg.</td>
<td>42.8 mg.</td>
<td>&quot;</td>
<td>As for Fraction 3</td>
</tr>
<tr>
<td>10</td>
<td>Chloroform</td>
<td>500 cc.</td>
<td>4.3 mg.</td>
<td>14.1 mg.</td>
<td>&quot;</td>
<td>Discard</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td>96.4 mg.</td>
<td>209.7</td>
<td></td>
</tr>
</tbody>
</table>

melting point depression was observed when mixed with authentic dehydroisoandrosterone acetate (m.p. 167–169°).

No other crystalline material could be obtained from the buffer-hydrolyzable \( \beta \)-17-ketosteroid fraction.

**Fractionation of “a”-17-Ketosteroids Released by Buffer Hydrolysis**—The “a” ketosteroids (containing 86 mg. of assayable 17-ketosteroid and weighing 213 mg.) were dissolved in 50 cc. of 1:1 pentane-benzene mixture and placed on an adsorption column of 67 gm. of aluminum oxide (25 mm. in diameter and 150 mm. in height, alumina adsorption, Fisher Scientific
Company). The first chromatogram achieved a separation of the "α"-ketosteroids into ten fractions (Table V). All fractions gave a positive test with tetranitromethane for the presence of unsaturation.

Fraction 2 (Table V) was crystallized from ethanol-pentane and yielded 5.8 mg. of a white crystalline compound melting at 156–158°. This compound gave an infra-red spectrophotogram identical with that of 3β-chloro-Δ4-androstenone-17 (m.p. 155–157°).

The other fractions could not be crystallized even after subjection to further fractionation with Girard's reagent and by chromatography (Table V). The fractions obtained in these attempted purifications were again tested with tetranitromethane and all gave strongly positive tests for unsaturation.

DISCUSSION

The observations that glucuronic acid and steroid gluconides have a pK value between pH 3 and 4.5 and that estriol glucuronide may readily be removed from butanol (19) by aqueous solutions at pH above 6 tend to indicate that the conjugated steroids that have a preferential solubility in the aqueous phase at pH above 3 are glucuronides. On the other hand, the fact that steroid sulfates in butanol solutions are not readily removed by aqueous alkalis (8, 9, 20) is indicative of the sulfate conjugation of the buffer-hydrolyzable ketosteroids, whose removal from urine by butanol is effected independently of pH.

The sulfate nature theory for the buffer-hydrolyzable ketosteroid conjugates is supported by two other sets of observations reported in this paper: (1) Hydrolysis of urines with glucuronidase neither influenced nor was influenced by the buffer hydrolysis. (The spleen glucuronidase preparations failed to hydrolyze pure steroid sulfates.) (2) The model experiments with pure steroid sulfates showed a lability to buffer hydrolysis of dehydroisoandrosterone sulfate and of cholesterol sulfate. An earlier observation that the presence of large amounts (15 gm. per cent) of sodium sulfate excerts a profound inhibitory effect on the buffer hydrolysis is also readily reconciled with the theory of a sulfate nature of the buffer-hydrolyzed ketosteroid conjugates.

The data presented in this paper indicate the necessity for buffer hydrolysis of steroid unsaturation (probably in a Δ5 position to an esterified alcohol group on C3). The importance of the steric relationships of the esterified hydroxyl group is not indicated in these studies. While it has been demonstrated that sulfates of 3β-hydroxy unsaturated steroids are hydrolyzed by the buffer technique, no 3α-hydroxy ketosteroids (i.e. with

*This analysis was kindly carried out by Dr. Konrad Dobriner of the Sloan-Kettering Institute for Cancer Research.
HYDROLYSIS OF CONJUGATED STEROIDS

a 3-OH trans to the C\textsubscript{10} methyl group) were demonstrable in the "α" fraction of the buffer-hydrolyzed urine. At least a part of this "α" fraction has been shown to be a non-hydroxyl artifact (3β-chloro-Δ\textsubscript{4}-androstenone-17).

It has been demonstrated that the lower β-hydroxy ketosteroid value (4.6 per cent) obtained following hydrolysis with hydrochloric acid, as compared to the relatively high proportion of β-hydroxy ketosteroids (14 per cent) obtained by buffer hydrolysis, is due to the destructive influence on the 17-ketosteroids of the HCl treatment (1). In view of the fact that 3β-chloro-Δ\textsubscript{4}-androstenone-17 was also isolated from the buffer-hydrolyzed ketosteroids, it is probable that there is an even greater amount of β-hydroxy unsaturated ketosteroids in the urine than that observed in the buffer hydrolysis experiments.

The importance of BaCl\textsubscript{2} in the buffer hydrolysis (2) was not supported by our experiments. The relative unimportance of urea in this hydrolysis is supported by the recent finding in our laboratory\textsuperscript{3} that the buffer hydrolytic technique may be directly applied to urine.

The ketosteroid conjugates of normal male urine which are not hydrolyzed by either the glucuronidase or buffer hydrolytic procedures probably consist, at least in large part, of saturated ketosteroid sulfates. The possibility of other types of conjugating substances cannot, however, be eliminated.

The isolation of about 8 per cent of the "total" urinary ketosteroids as dehydroisoandrosterone represents an amount of this steroid considerably greater than that heretofore reported for normal male urine (21).

SUMMARY

1. Factors affecting the preparation of a butanol extract of the conjugated ketosteroids in urine and those influencing their hydrolysis by buffers have been studied.

2. These studies led to the development of a procedure for the buffer hydrolysis of a specific group of conjugated ketosteroids in urine.

3. The ketosteroids thus released constitute about one-fourth the amount released by HCl hydrolysis and contain about 14 per cent of the "total" 17-ketosteroid content of urine as β-hydroxy compounds.

4. Evidence is presented to indicate that the ketosteroids released by the buffer hydrolytic procedure are unsaturated and are conjugated as sulfates.

5. The isolation of 80 mg. of dehydroisoandrosterone and of 6 mg. of 3β-chloro-Δ\textsubscript{4}-androstenone-17 from 100 liters of normal male urine is described.
We wish to acknowledge the technical assistance of Saranette Frank in many phases of this problem.

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HYDROLYSIS OF URINARY CONJUGATED 17-KETOSTEROIDS BY ACETATE BUFFER
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