THE ESTIMATION OF HYDROXYSTEROIDS*

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(Received for publication, August 19, 1954)

A number of methods have been described for the quantitative estimation of steroids having rather specific groups, e.g. 17-ketone groups, or \( \alpha \)-ketolic side chains at C-17. Procedures with such high specificity have been extremely useful in studies of steroid metabolism. They have been particularly valuable for the estimation of such compounds in urinary extracts and in the fractionation of these extracts. There are, however, steroids present in such extracts which, lacking these specific groupings, are not measured by any of the commonly employed methods. One large class of compounds of this type is that consisting of non-ketonic steroid alcohols. Over twenty compounds of this type have been isolated from urine, and included in this group are metabolites of androgens and adrenal cortical steroids as well as the major recognized metabolite of progesterone, which is a non-ketonic steroid alcohol, pregnane-3\( \alpha \),20\( \alpha \)-diol (1). A method for the quantitative estimation of such hydroxysteroids should prove useful for the evaluation of the function of the endocrine organs and in systematic fractionations of this group of metabolites.

Several methods have previously been described for the estimation of non-ketonic steroid alcohols (1-8) and have been applied to urinary extracts (9-13). The results of these relatively few studies indicate that further investigation of this fraction should prove profitable.

The method reported in this paper is based on the formation of acetate esters of the steroid hydroxyl groups, followed by the conversion of the acetates to acetohydroxamic acid with alkaline hydroxylamine. The acetohydroxamic acid forms a purple complex with ferric ion. The intensity of the resulting color is then used as a measure of the original con-

* This work was supported by grants from the National Cancer Institute, United States Public Health Service (grant No. C-1393(C,C2)), the American Cancer Society upon recommendation by the Committee on Growth of the National Research Council, the Jane Coffin Childs Memorial Fund for Medical Research, and an Institutional Grant from the American Cancer Society to the Massachusetts General Hospital. Preliminary reports of portions of this work were presented at the Laurentian Hormone Conference, Shrewsbury, Massachusetts, May 31–June 4, 1953 (1), and the Thirty-eighth meeting of the American Society of Biological Chemists, Atlantic City, April 12-16, 1954. This is Publication No. 332 of the Cancer Commission of Harvard University.
tent of hydroxyl groups. Quantitative methods based on the formation of hydroxamic acids have been reported for the estimation of anhydrides such as acetyl phosphate (14), glycerol esters (15–17), water-soluble esters (18), and amides (19). The use of such a method for hydroxyl groups in lipides was suggested by Hill (15), who did some preliminary work on the problem. Recently Zaffaroni has reported the utilization of these reactions for the determination of 11-deoxycorticosterone acetate and has suggested the more general use for steroid alcohols (20).

The experiments described here serve to elucidate the optimal conditions for the application of these reactions to the estimation of hydroxysteroids. The resulting method has been applied to a number of pure steroid alcohols and to urinary extracts. The results of these studies are presented.

**EXPERIMENTAL**

Three reactions are involved in the estimation of hydroxysteroids by the method described here. The first is the acetylation of the hydroxyl groups. The second is the cleavage of the acetoxyl groups with hydroxylamine to form acetohydroxamic acid. The third reaction is the formation of the purple ferric ion-acetohydroxamic acid complex. Each of these reactions will be considered separately.

*Acetylation of Hydroxysteroids*—Only one basic method for the acetylation of steroid hydroxyl groups has been investigated, the pyridine-catalyzed reaction with acetic anhydride. Relatively few variations of this method have been studied, since quantitative acetylation with this reagent is not difficult and can be achieved under a variety of conditions. The reagent used throughout this work has been a freshly prepared mixture of equal volumes of acetic anhydride and pyridine. Quantitative acetylation, determined by conversion to acetohydroxamic acid as described later, was achieved in 1 hour on a boiling water bath with 0.2 ml. of this reagent for 0.5 to 5 μeq. of hydroxysteroid (0.2 to 2 mg. of cholesterol). Most hydroxyl groups studied were quantitatively esterified in 10 minutes; however, the 3β-hydroxyl group of coprostanol required 60 minutes. Hence, the longer period has been chosen for routine use. It has been found, in agreement with the results of others, that pyridine-acetic anhydride fails to acetylate 11β-hydroxyl or tertiary 17α-hydroxyl groups.

Preliminary studies have indicated that it may be possible to acetylate certain groups selectively by varying the conditions for acetylation.

*Conversion of Acetoxyl Groups to Acetohydroxamic Acid*—The basic reagent used for this reaction is a solution of hydroxylamine in alcoholic potassium hydroxide. This is prepared by mixing alcoholic KOH with concentrated aqueous hydroxylamine hydrochloride and centrifuging to remove precipitated KCl. The effects of variations in solvent, concentra-
of KOH, concentration of hydroxylamine, and reaction time have been investigated with cholesteryl acetate, 11-deoxycorticosterone acetate, and allopregnane-3β,16α,20β-triol triacetate as model compounds.

Solvent—Aqueous methanol and aqueous ethanol have been used as solvents for this step of the procedure; however, ethanol is preferable, since most of the compounds studied are more readily soluble in ethanol than in methanol. The concentration of water in the ethanol has been varied from 3 to 15 per cent without significant effect on the course of the reaction. Therefore, the concentration has been kept to the minimum necessary to contain the hydroxylamine hydrochloride added in preparation of the reagent. It has been found that dissolving the steroid in a minute volume of diethyl ether before adding the alcoholic hydroxylamine-potassium hydroxide reagent greatly facilitates the solution of the steroid acetates in the reagent.

Concentrations of KOH and NH₂OH—The effects of varying the concentrations of KOH and NH₂OH independently have been studied. It has been found that the rate of the reaction is increased by increases in KOH concentration. Increases in NH₂OH concentration lead to increased stability of the acetohydroxamic acid. Pure acetohydroxamic acid does not undergo measurable decomposition in 10 minutes in the reagent finally selected for use and described below. When the hydroxylamine concentration is decreased by 50 per cent, 20 per cent of pure acetohydroxamic acid is destroyed in the same period of time at a rate independent of the concentration of alkali. Extensive studies of the effects of variations in the concentrations of the two reagents were not necessary. Reagents were initially tried which contained lower concentrations of hydroxylamine and potassium hydroxide. The concentrations were then increased progressively by large increments, which led to increased rates of reaction and stability of the hydroxamic acid. The concentrations finally selected for the reagent were those which gave quantitative yields of hydroxamic acid in a reasonable period of time, 10 minutes. No advantage could be foreseen in the use of higher concentrations.

Formation of Ferric-Acetohydroxamic Acid Complex—The final step in the series of reactions constituting the determination of hydroxyl groups is the formation of the colored complex of the hydroxamic acid with ferric ion. In the actual procedure, it is necessary to neutralize the potassium hydroxide from the preceding step, since the complex is stable over a relatively narrow pH range, 1.0 to 1.4 (18). The optimal amounts of acid and ferric chloride were determined empirically and are contained in the reagent described below. Lower concentrations of ferric chloride fail to yield maximal color with acetohydroxamic acid, and higher concentrations lead to unnecessarily high blanks. The concentration of hydrochloric
acid is that found to yield optimal color intensity and stability. Variations of up to 5 per cent in the concentration of hydrochloric acid are without measurable effect, but greater variations in either direction markedly decrease the color intensity of the complex.

Hill suggested the use of ferric perchlorate in place of ferric chloride and either perchloric or nitric acid along with the hydrochloric acid (15, 16). These substitutions were tried and did not in any way improve the complex formation under the conditions used here. Likewise the separate addition of the acid and ferric chloride did not offer any advantage.

Procedure for Estimation of Hydroxysteroids

The experiments outlined above have served to define conditions necessary for quantitative results in each step of the procedure. The next portion of the paper will describe the method for estimating hydroxysteroids which has resulted from the utilization of the information gained from the studies of these reactions.


Anhydrous pyridine. Merck, reagent, or Mallinckrodt, analytical reagent, refluxed over BaO and redistilled; b.p. 114°.

Acetic anhydride-pyridine reagent. Prepared by mixing equal volumes of acetic anhydride and anhydrous pyridine immediately before use.

Methanol, C. P. National Aniline.

Diethyl ether. Mallinckrodt, analytical reagent.

Hydroxylamine hydrochloride solution. Prepared by dissolving 56 gm. of hydroxylamine hydrochloride (Eastman or Matheson) in 100 ml. of distilled water.

Alcoholic potassium hydroxide solution. Prepared by dissolving 15.8 gm. of potassium hydroxide pellets (Mallinckrodt, analytical reagent, 85 per cent KOH) in 100 ml. of 95 per cent ethanol. Stable for at least 1 month when kept at 5°.

Alkaline hydroxylamine reagent. Prepared by mixing 7.5 volumes of alcoholic potassium hydroxide solution and 1 volume of hydroxylamine hydrochloride solution and centrifuging to remove the precipitate. Must be prepared immediately (not more than 15 minutes) before use.

Ferric chloride-hydrochloric acid reagent. Prepared by dissolving 1.7 gm. of FeCl₃·6H₂O (Mallinckrodt, analytical reagent) in 100 ml. of dilute HCl (6.5 ml. of concentrated HCl diluted to 100 ml. with H₂O).

Cholesterol standard. 1.00 mg. of cholesterol (m.p. 146–147°) per ml. in absolute ethanol.

Analytical Method—The samples to be analyzed, along with cholesterol standards, which should contain 0.5 to 5 μeq. of acylable hydroxyl, are ob-
tained dry in test-tubes calibrated for use as cuvettes in a photometer. 1
0.2 ml. of the freshly prepared acetic anhydride-pyridine reagent is added
to each tube, including empty ones to serve as blanks. The tubes are ro-
tated to dissolve the samples, stoppered with corks wrapped in aluminum
foil, and heated in a boiling water bath for 1 hour. The acetic anhydride
and pyridine are then removed by evaporation under a stream of air in the
water bath. When the odors of acetic anhydride and pyridine have dis-
appeared, 0.1 ml. of methanol is added to each tube. The tubes are rotated
to spread the methanol over the surface previously in contact with acetic
anhydride and pyridine. The methanol is subsequently evaporated on a
boiling water bath. This operation removes the last traces of the reagents.

Each acetylated sample is then dissolved in 0.2 ml. of diethyl ether, and
1 ml. of alkaline hydroxylamine reagent is added. The tubes are shaken
and allowed to stand at room temperature for 10 minutes. 3 ml. of the
ferric chloride-hydrochloric acid reagent are then added to each tube to
neutralize the base and form the colored complex. Each tube must be
stopped and shaken immediately after this reagent is added to avoid
reduction of ferric ion by alkaline hydroxylamine. 2

At this point, the alcohol content of the solution is about 20 per cent;
hence, most steroids and other lipides present will precipitate, causing
cloudiness. This is overcome by adding 4 ml. of diethyl ether to each tube
and shaking for a short period to extract the aqueous phase. This pro-
cedure, in addition to removing turbidity, extracts any lipide-soluble pig-
ment from the aqueous solution. It is unnecessary to remove the ether
layer, since it is above the light path of the photometer.

When 30 minutes have elapsed after the addition of the ferric chloride-
hydrochloric acid reagent, the tubes are read in the photometer at 560
mμ. 3 The absorption spectrum for the colored ferric-acetohydroxamic
acid complex was determined with the Cary spectrophotometer and the
peak absorption found to be at 527 mμ. However, the peak is quite broad,
and the absorption at 560 mμ is only slightly lower than at the peak. The
blank color, due to ferric ion absorption, is below 400 mμ, but the absorp-
tion at 530 mμ is appreciable when the Coleman junior spectrophotometer

1 Calibrated Pyrex test-tubes (13 X 100 mm.) were used in this work.
2 Krogh-Keys syringe pipettes have been found useful for the addition of the
ferric chloride-hydrochloric acid reagent, since delivery from these pipettes is ac-
companied by satisfactory mixing. The tubes are then stoppered and shaken after
the reagent has been added to all tubes in a series.
3 Immediately after adding the ferric chloride-hydrochloric acid reagent, erratic
color formation occurs in many tubes, owing probably to the formation of some
ferric hydroxide. This color fades quite rapidly after mixing and is completely ab-
sent after 30 minutes standing. The ferric-acetohydroxamic acid complex is reason-
ably stable under these conditions, fading at the rate of 2 per cent per hour.
is employed. The absorption is about 50 per cent lower at 560 μ. For this reason, the latter wave-length has been used in the determinations. It is likely that a wave-length closer to the absorption maximum might be better with photometers having greater resolving power.

The readings of samples and standards are corrected for the blank reading, and the microequivalents of acylable hydroxyl per sample are calculated by comparison with standards of cholesterol run at the same time.

**Yields of Chromogen from Pure Steroids**

*Steroid Acetates*—Cholesteryl acetate has been analyzed by the above method, with the omission of the acetylation steps. By comparison with pure acetohydroxamic acid, it was found to yield the ferric-acetohydroxamic acid chromogen quantitatively. Since cholesteryl acetate is readily available, it has been used as the standard against which other steroid acetates and steroid alcohols have been compared. The yields of chromogen from allopregnane-3β,16α,20β-triol triacetate and from deoxycorticosterone acetate were 91 per cent, compared with cholesteryl acetate on an equivalent basis. These low yields were increased by about 10 per cent upon acetylation. This indicates that the samples were not pure and had probably undergone some hydrolysis on standing. All three steroid acetates showed a linear relationship between weight and absorbance in the region studied, from 0.5 to 5 μeq.

*Steroid Alcohols*—A number of pure steroid alcohols have been subjected to the entire analytical procedure and their percentage yields of acetohydroxamic acid calculated on an equivalent basis by comparison with cholesteryl acetate. These compounds were selected to contain hydroxyl groups in the positions and with the configurations usually encountered in naturally occurring steroids. The yield of chromogen from each of these compounds is presented in Table I. All values are means of two or more sets of determinations carried out with at least two different levels of steroid, usually 1 and 3 μeq. The relationship between absorbance and quantity was found to be linear through the origin for all compounds studied. Statistical analysis of the data indicates that there is no significant difference between the yields of chromogen from the various compounds studied. The mean yield for all compounds studied is 96 per cent, with a standard deviation of ±3.7 per cent. The results on progesterone show that a hydroxyl group is necessary for chromogen formation.

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4 As demonstrated below, all hydroxysteroids yield the same amount of chromogen per microequivalent of acylable hydroxyl; hence, any pure hydroxysteroid can be substituted for cholesterol as the standard.

5 The source of the chromogen in this case is probably Δ^1-pregnen-3β-ol-20-one, a known contaminant of commercial progesterone (21).
Estimation of Hydroxysteroids in Neutral Urinary Extracts

In the application of the above method to neutral extracts of acid-hydrolyzed urine, it was found that these extracts, prior to acetylation, contain material which reacts with the alkaline hydroxylamine reagent to form products that subsequently yield chromogen with the ferric chloride reagent. This material presumably consists of esters, since it is substantially reduced by saponification of the extract with 1 N KOH in methanol prior to analysis, and since the extracts do not react with ferric chloride to form chromogen when the reaction with hydroxylamine is omitted. Investigation of the nature of this material is under way, since it may be partly composed of esterified steroid alcohols, either excreted in this form or formed during the processing of the extracts. Until its nature is more clearly defined, however, it seems preferable to correct for its contribution to the final chromogen formation. This has been done routinely by running duplicate samples of the urinary extracts through the analytical procedure without the acetylation. The absorbance of this sample, referred to as the "preformed ester blank" is subtracted from the absorbance of the corresponding acetylated sample. This procedure also corrects for any pigment not extracted by the ether in the analytical procedure. The magnitude of this preformed ester blank is quite variable from extract to extract. In most cases it amounts to about 10 per cent of the value for the acylable material; however, in the extracts from a few individuals the values were considerably higher.

In the analysis of urinary extracts, the extracts, cholesterol standards,

<table>
<thead>
<tr>
<th>Table 1</th>
</tr>
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Percentage Yield of Acetohydroxamic Acid Chromogen from Various Steroids

<table>
<thead>
<tr>
<th>Compound</th>
<th>Yield* per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>99</td>
</tr>
<tr>
<td>Coprostanol</td>
<td>94</td>
</tr>
<tr>
<td>Cholestanol</td>
<td>100</td>
</tr>
<tr>
<td>Epicoprostanol</td>
<td>98</td>
</tr>
<tr>
<td>Epicholestanol</td>
<td>95</td>
</tr>
<tr>
<td>Testosterone</td>
<td>94</td>
</tr>
<tr>
<td>Pregnane-3α,20α-diol</td>
<td>94</td>
</tr>
<tr>
<td>Allopregnane-3β,16α,20β-triol.</td>
<td>97</td>
</tr>
<tr>
<td>Deoxycorticosterone</td>
<td>94</td>
</tr>
<tr>
<td>Progesterone</td>
<td>2†</td>
</tr>
</tbody>
</table>

* Based on comparison with the yield of chromogen from cholesteryl acetate.
† Calculated for a monohydroxy compound of molecular weight 314.
and blanks are run through all steps of the analytical procedure as described above, beginning with the acetylation. Duplicate samples of the extracts along with unacetylated blanks are run through the steps beginning with the addition of 0.2 ml. of ether, the acetylation steps being omitted. The acetylated blank, referred to as the "acetylation blank," usually has an absorbance about 0.02 above that of the unacetylated blank, referred to as the "reagent blank." A difference greater than this indicates a lack of purity of either the pyridine or acetic anhydride.

### Table II

**Daily Excretion of Urinary Neutral Acylable Lipides**

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age yrs.</th>
<th>Condition</th>
<th>Total µeq. per 24 hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>F.</td>
<td>24</td>
<td>Normal</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pregnancy (1st trimester)</td>
<td>194</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&quot; (3rd &quot; )</td>
<td>258</td>
</tr>
<tr>
<td>F.</td>
<td>50</td>
<td>Normal</td>
<td>102</td>
</tr>
<tr>
<td>&quot;</td>
<td></td>
<td>&quot;</td>
<td>96</td>
</tr>
<tr>
<td>M.</td>
<td>53</td>
<td>&quot;</td>
<td>150</td>
</tr>
<tr>
<td>&quot;</td>
<td>68</td>
<td>Prostatic carcinoma</td>
<td>114</td>
</tr>
<tr>
<td>&quot;</td>
<td>48</td>
<td>Carcinoma, breast</td>
<td>110</td>
</tr>
<tr>
<td>F.</td>
<td>55</td>
<td></td>
<td>178</td>
</tr>
<tr>
<td>&quot;</td>
<td>43</td>
<td>&quot;</td>
<td>40</td>
</tr>
<tr>
<td>M.</td>
<td>55</td>
<td>Rheumatoid arthritis</td>
<td>82</td>
</tr>
<tr>
<td>&quot;</td>
<td>34</td>
<td></td>
<td>125</td>
</tr>
<tr>
<td>F.</td>
<td>45</td>
<td>Addison’s disease</td>
<td>26</td>
</tr>
<tr>
<td>M.</td>
<td>60</td>
<td>Adrenal carcinoma</td>
<td>410</td>
</tr>
<tr>
<td>F.</td>
<td>43</td>
<td>&quot;</td>
<td>288</td>
</tr>
<tr>
<td>&quot;</td>
<td>21</td>
<td>Cushing’s disease</td>
<td>177</td>
</tr>
</tbody>
</table>

* Mean of five values obtained at various phases of the menstrual cycle (range 133 to 184 µeq.).

To calculate the microequivalents of acylable hydroxyl in the unknown, the following formula is employed,

\[
\mu \text{eq.}_{\text{unk.}} = \frac{U - R_a - (R_{pe} - R_r)}{S - B_a} \times \mu \text{eq.}_{\text{std.}}
\]

where, \(\mu \text{eq.}_{\text{unk.}}\) = microequivalents of acylable hydroxyl in the unknown

\(U\) = absorbance of unknown at 560 mp

\(B_a\) = " acetylation blank at 560 mp

\(B_{pe}\) = " preformed ester blank at 560 mp

\(B_r\) = " reagent blank at 560 mp

\(S\) = " hydroxysteroid standard at 560 mp

\(\mu \text{eq.}_{\text{std.}}\) = microequivalents of acylable hydroxyl in hydroxysteroid standard
In Table II are presented some representative results obtained by this method for the estimation of urinary neutral acylable lipides. These determinations were carried out in duplicate on aliquots of urinary extracts corresponding, in most cases, to about 1 per cent of a 24 hour urine specimen. The above formula was utilized for the calculations. These examples have been selected to demonstrate variations in the excretion of neutral acylable lipides with changes in the endocrine states of the patients.

Fig. 1 shows the effect of testosterone administration on the urinary excretion of neutral acylable lipides by a patient with rheumatoid arthritis.

![Graph showing the effect of testosterone administration on urinary excretion of neutral acylable lipides and 17-ketosteroids.](http://example.com/graph.png)

**Fig. 1.** The effect of intramuscular administration of testosterone on the urinary excretion of neutral acylable lipides (NAL) and ketosteroids in a male patient, 59 years old, with rheumatoid arthritis. DHA is the 17-ketosteroid value calculated as microequivalents of dehydroepiandrosterone per 24 hours. NAL represents the neutral acylable lipides as measured by the ferric-acetohydroxamic acid method.

The daily excretion of 17-ketosteroids (micromoles) as determined by a modification of the Zimmermann reaction (22, 23) is included for comparison. The administration of testosterone is accompanied by a marked increase in the excretion of materials measured by both methods; that is, 17-ketosteroids and neutral acylable lipides. The close correlation between the values for the two groups of compounds during administration of testosterone is consistent with the excretion of androsterone and etiocholan-3α-ol-17-one as the major urinary metabolites of this hormone.

**DISCUSSION**

The method presented here offers a simple and rapid method for the quantitative estimation of a group of urinary steroids not measured by more specific methods. Obviously this method has no intrinsic specificity.
for steroid alcohols, but will likewise measure other lipide-soluble alcohols. However, the good correlation between the daily excretion of neutral acylable lipides and the state of endocrine balance suggests that under most conditions the major portion of the urinary neutral acylable lipides is composed of hydroxysteroids. The extent to which non-steroidal compounds contribute to the values obtained by this method must await the fractionation and identification of the components of this fraction. Studies in this direction are now under way.

This method has several potential uses (1) in addition to its application for the estimation of urinary neutral acylable lipides. It has been employed in this laboratory for the determination of the number of acylable hydroxyl groups in pure compounds of unknown structure in which the approximate molecular weights were known. It has also been used to follow the course of hydrolysis of acetate esters of hydroxysteroids.

SUMMARY

1. A method is presented for the quantitative estimation of acylable hydroxyl groups in lipides.

2. The method has been applied to steroids possessing hydroxyl groups in the positions and with the configurations commonly encountered in urinary steroids. The yield of chromogen has been found to correspond to 96 per cent of the theoretical yield for all hydroxyl groups acetylated under base catalyzed conditions.

3. The urinary excretion of neutral acylable lipides has been found to correlate with the state of endocrine function, suggesting that the compounds measured are predominantly steroids.

The authors wish to express their thanks to the following for their generous gifts of compounds: Ciba Pharmaceutical Products, Inc. (deoxy-corticosterone acetate, progesterone, and testosterone), Dr. John D. Gregory (acetohydroxamic acid), Parke, Davis and Company (pregnanediol).

We also wish to express our thanks to Miss Gladys Ekman for the keto-steroid analyses and to Miss Electra Paskalides for the preparation of the urinary extracts.

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