THE HYDROLYSIS OF STEROID GLUCURONIDES WITH CALF SPLEEN GLUCURONIDASE∗

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It has long been recognized that the relatively severe acid hydrolysis necessary to release steroids from their conjugates in urine may result in considerable losses or transformations (3, 4). The isolation of steroid glucuronides (5-7) and of steroid sulfates (8, 9) from human urines has led to the belief that most, if not all, of the urinary steroid conjugates exist as glucuronides or as sulfates. The separate hydrolysis of these two types of conjugates seems to offer the greatest possibility of development of less drastic hydrolytic procedures than those employing strong acids at high temperatures. The development of such specific hydrolytic procedures was felt highly desirable in connection with our studies designed to permit the isolation of a greater number of urinary steroid conjugates than have hitherto been characterized. The concentration of a specific group of steroid conjugates could thus be readily followed.

The possibility of an enzymatic hydrolysis of conjugated steroids was first apparent in the observation of Cohen and Marrian in 1935 (10) that prolonged exposure of pregnancy urine to bacterial action resulted in a complete hydrolysis of the conjugated estrogens. Subsequent identification of a considerable portion of this estrogen as estriol glucuronide indicated the involvement of some bacterial glucuronidase in this hydrolysis. Shortly thereafter Patterson (11) used preparations of Escherichia coli to effect a hydrolysis of the conjugated estrogens in early pregnancy urines, thus obtaining estrogen concentrates which on colorimetric assay gave a considerably reduced amount of interfering background chromogen. In 1939 Fishman (12) reported on the ability of preparations of ox spleen β-glucuronidase to effect hydrolysis of pure sodium estriol monoglucuronidate. Rat liver preparations were subsequently used by Talbot et al. (13) and by Mason and Kepler (14) for the hydrolysis of C19- and C21-steroid glucuronides. In 1949 Buehler et al. (15) reported on the successful

*This investigation was supported by research grants from the National Institutes of Health, United States Public Health Service, and the Graduate School of the University of Minnesota. For the preceding papers on the conjugated steroids, see Cohen (1) and Bitman and Cohen (2).
use of a glucuronidase preparation of bacterial origin for effecting an efficient hydrolysis of steroid glucuronides. The experiments reported in this paper involve glucuronidase concentrates prepared from calf spleen and were based upon a desire to utilize a readily available and easily concentrated source of the enzyme. Studies on the hydrolysis of the conjugated corticoids, ketosteroids, and estrogens in urine and with pure sodium pregnanediol glucuronidate and sodium estriol glucuronidate with such glucuronidase concentrates were made, and quantitative procedures for the hydrolysis of urinary ketosteroids and corticoids were evolved.

Methods of Assay

Glucuronidase activity was measured by the procedure of Fishman et al. (16); 1 unit of glucuronidase is defined as the amount of enzyme which liberates 1 γ of phenolphthalein per hour from the substrate phenolphthalein glucuronide.

Urinary corticoids were assayed by the modified formaldehydogenic procedure of Corcoran and Page (17) as recommended by Mason and Sprague (18). Acid hydrolysis was effected by adjusting the urine specimens to pH 1.0 with concentrated HCl and subjecting to continuous extraction for 24 hours through 0.5 volume of CHCl₃ at room temperature (19).

17-Ketosteroids were determined on the neutral ketonic fractions of samples of urine prepared and assayed as previously described (4). The acid hydrolyses were effected by refluxing the urine specimens with 15 per cent by volume of concentrated HCl for 10 minutes.

The estriol and estrone fractions of pregnancy urine were approximately separated by the procedure of Cohen and Marrian (20). The estrogen concentrates were assayed by the method of Stevenson and Marrian (21).

Glucuronic acid was determined by the method of Maughan, Evelyn, and Browne (22).

EXPERIMENTAL

Preparation of Glucuronidase Concentrates—Calf spleen is a very potent tissue source of β-glucuronidase and was therefore used as the enzyme source material in these investigations. Enzyme concentrates are prepared from dried defatted calf spleen¹ by a procedure based on that suggested by Fishman and Talalay (23).²

100 gm. of spleen are homogenized once with 400 cc. and then four times

¹ The calf spleen used as the initial source of glucuronidase concentrate preparations was obtained in large part from the VioBin Corporation, Monticello, Illinois. One 5 pound lot of dried spleen was prepared in our laboratory by extracting ground fresh calf spleen several times with acetone and then drying and powdering the insoluble portion.

² Personal communication from Dr. Fishman.
with 200 cc. volumes of 0.1 N acetate buffer, pH 4.5. After each homogenization the mixture is centrifuged and the supernatant clarified by filtration. The combined filtrate is precipitated by half saturation with ammonium sulfate. The precipitate is removed by centrifugation, dissolved in about 200 cc. of water, and dialyzed against cold tap water for 16 to 24 hours. The precipitation with \((\text{NH}_4)_2\text{SO}_4\) is repeated and the precipitate dissolved in a small volume of water (30 to 100 cc.). A summary of the enzyme concentrations typically effected by this procedure is shown in Table I. It is seen that a 60-fold increase in activity is effected with a loss of about 30 to 40 per cent of the original total activity. The greatest losses occurred during the dialyses, and were often considerable in the second dialysis (Fraction 6, Table I); this second dialysis is therefore omitted in the routine preparations. The glucuronidase concentrate is refrigerated when not in use; under these conditions it is stable for at least 2 months.

Kerr, Levvy, and Walker (24) have reported that more potent glucuronidase extracts can be prepared from mouse livers with citrate buffer than with acetate buffer. Attempts were therefore made to prepare glucuronidase concentrates from spleen with citrate instead of acetate buffer in the initial extractions. The great difficulty in clarifying the citrate buffer extract and subsequent solutions from fatty material, and the high blank formaldehydogenic values obtained with such extracts, soon led to the abandonment of these attempts.

Blank Control Assays—These assays were carried out by dissolving enzyme concentrate (about 5000 units) in 0.1 N buffer, pH 4.5, and incubating at 38° for 1 to 5 days. After incubation the solutions were subjected to the extraction and assay procedures used for the various steroids studied in this investigation.
Corticoids—A considerable formaldehyde color is obtained in the control blank corticoid assay. Since the enzyme-released corticoid determinations are carried out on about 25 cc. of urine (see below), the calculation of formaldehydogenic corticoids per liter of urine may involve a 10 to 50 per cent error (2 to 3 mg. of "corticoids" per liter of urine). These enzyme blank assays are fairly constant for any one batch of glucuronidase preparation. A blank correction factor is therefore determined for all new batches of glucuronidase preparations. All the corticoid assays involving enzyme hydrolysis of the urine specimens reported in this investigation are corrected for these blank control assays.

Ketosteroids and Estrogens—No significant ketosteroid or estrogen chromogen was detected for the enzyme blank controls when up to 20,000 units of β-glucuronidase were used from several different enzyme batches.

Pregnanediol—When pregnanediol assays were carried out by the colorimetric procedure of Talbot et al. (25) on ether extracts of enzyme blank control samples, a considerable amount of color was obtained. The use of pregnanediol assays in studies of glucuronidase hydrolysis of pure pregnanediol glucuronide thus appeared to involve preliminary purification studies to eliminate this interfering chromogen. Fortunately, however, assays on the liberated glucuronic acid could readily be effected without such interference and these were substituted for pregnanediol assays in studies on the enzyme hydrolysis of pregnanediol glucuronide.

Glucuronic Acid—No significant chromogen was apparent in the enzyme blank controls when these were subjected to the glucuronic acid assay procedure (22). In order that the released glucuronic acid could be used as an index of the degree of hydrolysis of pregnanediol glucuronide, the following relatively simple procedure for their separation from each other was devised.

Separation of Free Glucuronic Acid from Pregnanediol Glucuronide

The aqueous solutions of hydrolyzed pregnanediol glucuronide are adjusted to pH 2 to 3 and extracted four times with 0.25 volume of butanol. Glucuronic acid assays are then carried out on the residues of suitable aliquots of the butanol and aqueous fractions. The effectiveness of such a procedure was demonstrated in a series of experiments in which samples of pregnanediol glucuronide (NaPG) and of glucuronic acid (GA) were added separately or together to 50 to 100 cc. of water and then subjected to the fractionation procedure outlined above. In one such experiment, for example, a solution of NaPG containing the equivalent of 650 γ of glucuronic acid resulted in a GA assay of 600 γ in the butanol extract and none in the aqueous extract, while a solution of 2.0 mg. of glucuronic acid resulted in its quantitative appearance in the aqueous extract. A
mixture of NaPG (containing an equivalent of 650 γ of GA) + 500 γ of glucuronic acid yielded 480 γ of GA in the aqueous extract and 640 γ in the BuOH extract. These data, as well as those obtained in several similar experiments, indicate the separation of free glucuronic acid into the aqueous phase and of the pregnanediol glucuronide into the butanol phase in these partitions.

Effect of Preliminary Sterilization of Urine Prior to Enzyme Hydrolysis

Since only urine specimens collected under very rigid conditions are relatively free of bacterial contamination, it was thought desirable to determine the effect of such contamination on the steroid content of urines hydrolyzed with preparations of spleen glucuronidase. The presence of bacterial glucuronidase would tend to speed up the rate of glucuronide hydrolysis but to have no effect on the maximum yield of glucuronidase-liberated steroids. On the other hand, the demonstration of ketosteroid transformations by bacterial enzymes (26) suggested that a reduction of assayable steroids might occur as a result of bacterial action. Experiments involving ketosteroid and corticoid assays on glucuronidase-hydrolyzed urine specimens indicated the possibility of both types of bacterial contamination.

Experiments were performed in which 25 to 50 cc. aliquot samples of pooled male urine were (a) boiled for 10 minutes, (b) filtered through a 02 Selas filter,³ and (c) used as collected (no preservative was employed during the collection of the urine). The urine samples were then brought to 0.1 N with respect to acetate buffer of pH 4.5 by the addition of a suitable amount of 5 to 10 N buffer, 4000 to 5000 units of glucuronidase concentrate were added to each, and the samples were analyzed for free steroids after incubation at 38° for 16 to 40 hours. For three to five urines thus studied, preliminary sterilization of the urines resulted in marked increases in the amounts of glucuronidase-released steroids. Thus, for example, 50 cc. specimens from one batch of pooled male urine (Batch MUI14) hydrolyzed with 4000 units of the glucuronidase preparation for 40 hours showed that the non-sterilized urine released corticoids and ketosteroids equivalent to 6 and 5.1 mg. per liter of urine respectively. After urine sterilization by either boiling or filtering through the Selas filter, corticoid and ketosteroid values of 16 and 7.5 mg. per liter respectively were obtained. These experiments do not indicate whether the sterilization procedures effect a removal of a glucuronidase inhibitor or of a steroid-destroying agent. In the other two experiments, preliminary boiling of the urine specimens caused a

³ This filter was obtained from the Selas Corporation of America, Philadelphia, Pennsylvania, and removes particles of about 0.85 μ diameter, including most bacteria.
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decrease in the rate but not in the maximum amount of glucuronidase hydrolysis of conjugated corticoids. This is interpreted as indicating a destruction of bacterial glucuronidase by the boiling.

Boiling approximately neutral urine specimens for 10 minutes was found in itself to cause no measurable release of corticoids or ketosteroids, nor to reduce the maximum amounts of the steroids subsequently released by hydrolysis with preparations of spleen glucuronidase. The stability of the ketosteroids and corticoid glucuronides in urine to heat at near neutral pH is further indicated (a) by the similarity in amounts of ketosteroids and corticoids released by the glucuronidase preparation for boiled and for Selas-filtered urines, and (b) in the failure of a 2 to 4 hour heating in a boiling water bath at pH 5.0 to influence the amount of subsequent glucuronidase-released ketosteroids (2) or corticoids (27). All of the enzyme hydrolysis experiments carried out on urine specimens reported in this paper involved a preliminary boiling of the urines for 10 minutes.

Factors Affecting Hydrolysis of Steroid Glucuronides by Spleen Glucuronidase

25 cc. aliquots of pooled urine from normal males were used for the studies on corticoids; 50 cc. aliquots of urine from a similar source were used for the studies on ketosteroids. Pregnancy urine (of about the 8th month of pregnancy) was used for the studies on urinary estrogens. The samples of sodium pregnanediol glucuronidate and of sodium estriol glucuronidate (NaEG) used in these studies were analytically pure. Enzyme hydrolyses were carried out at about 38° in all cases.

pH—In Fig. 1 are shown the effects of pH on the hydrolysis of a number of steroid conjugates by the glucuronidase concentrate. The urines were boiled, adjusted to the desired pH, and then a sufficient volume of concentrated acetate buffer to give a final buffer concentration of 0.1 N was added. The pure steroids were dissolved in 25 to 50 cc. of 0.1 N acetate buffer of the desired pH. Enzyme concentrate was then added to the buffered solutions and the specimens extracted and assayed for the freed steroids after incubation. In no instance was a significant change in pH observed at the end of the incubation time.

It is seen that the optimum pH for the enzyme hydrolysis of all the steroid conjugates examined is about 5.0, being somewhat lower for urinary estriol and for pure pregnanediol glucuronide and somewhat higher for the urinary corticoid glucuronides. A urine pH of 5.0 was selected for all subsequent glucuronidase hydrolysis experiments, since this pH permitted the separate assay of a number of steroid types in a single enzyme-hydrolyzed urine specimen.

Enzyme Concentration—In Fig. 2 is shown the effect of glucuronidase concentration on the rate of steroid release from the corresponding conju-
gates. The hydrolyses were carried out on samples made 0.1 N with respect to pH 5.0 acetate buffer, as described above. Various amounts of glucuronidase concentrate were then added and the freed steroids determined after incubation. It is seen that the sodium pregnanediol glucuronidate is more rapidly hydrolyzed than any of the other steroid conjugates tried, being completely hydrolyzed under the conditions of the experiment by 1000 units of glucuronidase concentrate in 4 hours. Conjugated corticoids, estriol, and ketosteroids in order are hydrolyzed at diminishing rates. The similarity in the rate of hydrolysis for the estriol glucuronide in urine and

![Graph](http://www.jbc.org/)

**Fig. 1.** Effect of pH on the hydrolysis of steroid glucuronides by calf spleen β-glucuronidase; Curve 1, corticoids liberated from urine Batch MU16 by 2000 glucuronidase units in 16 hours; Curve 2, ketosteroids liberated from urine Batch MU16 by 4000 glucuronidase units in 16 hours; Curve 3, glucuronic acid liberated from NaPG containing the equivalent of 620 γ of glucuronic acid by 500 glucuronidase units in 3½ hours; Curve 4, estriol liberated from 50 cc. of pregnancy urine (Batch LPU11) by 2000 glucuronidase units in 64 hours. For further details, see the text.

for solutions of pure sodium estriol glucuronidate is also apparent from these data.

*Time of Incubation*—The effect of the time of incubation on the hydrolysis of conjugated steroids by the glucuronidase concentrate is illustrated in Fig. 3. Control experiments have revealed that only a negligible degree of steroid conjugate hydrolysis occurs in boiled urines incubated at pH 4.5 for up to 14 days. The relative rates of hydrolysis of the various steroid glucuronides indicated in the enzyme concentration studies (see Fig. 2) are confirmed.

Preliminary studies on the hydrolysis of the various types of estrogen in pregnancy urine indicate that the free estrogens appearing in the “weakly phenolic” (20, 28) fraction of hydrolyzed urine are liberated from their precursors at a rate three to four times greater than is estriol. Thus, for example, the incubation of 800 cc. of pregnancy urine (Batch LPU12) with
Fig. 2. Effect of glucuronidase concentration on the hydrolysis of steroid glucuronides; Curve 1, corticoids released from urine Batch MUI after 16 hours incubation at 38°; Curve 2, ketosteroids released from urine Batch MU17 after 16 hours incubation at 38°; Curve 3, glucuronic acid released from NaPG containing the equivalent of 260 γ of glucuronic acid after 4 hours incubation at 38°; Curve 4, estriol released from 100 cc. of pregnancy urine (Batch LPU18) after 16 hours incubation at 38°; Curve 5, estriol released from NaEG containing the equivalent of 570 γ of estriol after 16 hours incubation at 38°. For further details, see the text.

Fig. 3. Effect of incubation time (38°) on the hydrolysis of steroid glucuronides by calf spleen β-glucuronidase concentrate; Curve 1, corticoids released from urine Batch MU20 by 2000 units of glucuronidase; Curve 2, ketosteroids released from urine Batch MU27 by 10,000 units of glucuronidase; Curve 3, glucuronic acid released from NaPG containing the equivalent of 270 γ of glucuronic acid by 500 units of glucuronidase; Curve 4, estriol released from 50 cc. pregnancy urine (Batch LPU18) by 3000 units of glucuronidase; Curve 5, estriol released from NaEG containing the equivalent of 570 γ of estriol by 2000 units of glucuronidase. For further details, see the text.
8500 units of glucuronidase concentrate for 4 hours resulted in the liberation of 1240 \( \gamma \) of "weakly phenolic" estrogen and 450 \( \gamma \) of estriol. Since no attempts to separate the estrone and estradiol in this former fraction have yet been made, it is not known whether the glucuronides of one or both of these steroids are involved in this relatively rapid hydrolysis.

**Summary of Procedures for Maximum Liberation of Corticoids and Ketosteroids from Urine by Spleen \( \beta \)-Glucuronidase Concentrates**

On the basis of these and a large group of similar studies on a number of urine specimens, the following procedures have been adopted for the glucuronidase hydrolysis of conjugated ketosteroids and corticoids in urine.

*Corticoids*—A 25 cc. urine aliquot is boiled for 10 minutes, adjusted to pH 5.0, and 2 per cent by volume of 5 N acetate buffer, pH 5.0, is added. After the addition of 2000 to 5000 units of the spleen \( \beta \)-glucuronidase concentrate, the samples are incubated at 37-38° for 2 to 4 days, at which time they are cooled and extracted with CHC13, etc., for corticoid assays.

*Ketosteroids*—A 50 cc. urine aliquot is similarly boiled and buffered and, after the addition of 10,000 units of the glucuronidase concentrate, the sample is incubated at 37-38° for 3 to 5 days. Then it is cooled, extracted, and assayed for ketosteroids.

When the steroid assay values thus obtained are greater than the equivalent of 20 mg. per liter of urine, then the enzyme hydrolysis is repeated with a smaller volume of urine and a longer period of incubation.

**Comparison of Amounts of Steroids Released from Urines by Glucuronidase and by HCl Hydrolysis**

A number of urine specimens have so far been assayed for corticoids and ketosteroids after hydrolysis by the glucuronidase procedure and by the standard acid procedures referred to under "Methods of assay." The results of a representative group of these assays are shown in Table II. In most of the enzyme assays reported in Table II, the values were checked as maximum values by assays on a second sample of urine to which a larger amount of enzyme had been added or a longer incubation time used.

Assays on samples from twelve different batches of pooled male urine, two of which are included in Table II, indicate that for normal male urine about 45 per cent (30 to 60 per cent) of the "total" ketosteroids (\( i.e., \) the amount of ketosteroids released by acid hydrolysis) is released by the glucuronidase hydrolysis. Trials with four pure steroid sulfates (the sodium sulfates of dehydroisoandrosterone, androsterone, estrone, and estradiol) have indicated a complete lack of hydrolytic influence on these

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4 Grateful acknowledgment is made to Dr. C. R. Scholz, Ciba Pharmaceutical Products, Inc., for the androgenic sulfates, to Dr. S. Cook of Ayerst, McKenna and
by the spleen glucuronidase preparations under conditions which resulted in maximum release of conjugated ketosteroids and estriol from urine. It is thus probable that at least a part of the conjugated ketosteroids not hydrolyzed by glucuronidase are conjugated as sulfates. A greater pro-

Table II

Urinary Ketosteroids and Corticoids Freed by Hydrolysis with Glucuronidase and with HCl

<table>
<thead>
<tr>
<th>Urine batch</th>
<th>Identification</th>
<th>Ketosteroids released by</th>
<th>Ratio of HCl to enzyme ketosteroids</th>
<th>Corticoids* released by</th>
<th>Ratio of HCl to enzyme corticoids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HCl</td>
<td>Glucuronidase</td>
<td>HCl</td>
<td>Glucuronidase</td>
</tr>
<tr>
<td>MU14</td>
<td>Normal male (pooled)</td>
<td>25.1</td>
<td>8.3</td>
<td>3:1</td>
<td>0.76</td>
</tr>
<tr>
<td>MU17</td>
<td>Normal male (pooled)</td>
<td>13.2</td>
<td>7.5</td>
<td>1.8:1</td>
<td>0.37</td>
</tr>
<tr>
<td>N. D.</td>
<td>Presurgery</td>
<td>4.9</td>
<td>2.4</td>
<td>2:1</td>
<td>0.35</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>Postsurgery</td>
<td>2.9</td>
<td>2.9</td>
<td>1:2.5</td>
<td>16.0</td>
</tr>
<tr>
<td>E. C.</td>
<td>Pregnant 8 mos. &quot; 8 &quot;</td>
<td>9.3</td>
<td>7.8</td>
<td>1.2:1</td>
<td>1.5</td>
</tr>
<tr>
<td>V. L.</td>
<td>&quot; 8 &quot;</td>
<td>13.7</td>
<td>9.5</td>
<td>1.4:1</td>
<td>1.6</td>
</tr>
<tr>
<td>R. D.</td>
<td>Patient with breast cancer on estrogen therapy</td>
<td>0.9</td>
<td>0.7</td>
<td>1.3:1</td>
<td>0.4</td>
</tr>
<tr>
<td>C. D.†</td>
<td>Female pseudo-hermaphrodite, aged 11 yrs. &quot; &quot;</td>
<td>76</td>
<td>33</td>
<td>2.3:1</td>
<td>0.4</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>Female pseudo-hermaphrodite, aged 11 yrs. on therapy of 100 mg. cortisone per day</td>
<td>11</td>
<td>7.3</td>
<td>1.5:1</td>
<td>0.9</td>
</tr>
</tbody>
</table>

* The steroids excreted are expressed as mg. per liter of urine for urine Batches MU14 and MU17 and as mg. per 24 hours urine specimen for the remainder of the urine batches.
† C. D. was a patient of Dr. Irvine McQuarrie of the Department of Pediatrics, University of Minnesota Hospitals, who kindly made urine and blood specimens available for study.

portion of the "total" 17-ketosteroids in pregnancy urine is hydrolyzed by glucuronidase (an average of 75 per cent for the three samples thus far examined). This finding is consistent with data reported by Buehler et al. (15) for urines hydrolyzed with glucuronidase preparations of bacterial origin.

Harrison, Ltd., for estrone sulfate, and to Dr. G. W. Holden of Charles E. Fro& and Company for a sample of sodium estradiol sulfate.
The amount of formaldehydogenic corticoids released by the glucuronidase preparation has been found, for eight batches of pooled male urine, to be 18 (range 12 to 27) times as great as that obtained after the hydrolysis at pH 1.0 with about the same ratio range shown for the other urine specimens examined. The only cortisone-treated patient that we have so far examined (C. D.) showed twice as great an increase in the glucuronidase-released corticoids as in the acid-released corticoids during this therapy. The high glucuronidase-to-acid-released corticoid ratio for all urine specimens thus far examined is not surprising in view of the mildness of the acid-hydrolytic procedures required to minimize destruction of the relatively labile corticoids.

**DISCUSSION**

Data reported in this paper indicate that about half of the HCl-hydrolyzable ketosteroids of normal male urine is released by hydrolysis with spleen glucuronidase concentrates. It has been shown that hydrolysis with 0.1 N acetate buffer affects about 25 per cent of the conjugated ketosteroids in such urines (2). Since neither of these procedures hydrolyzes the sulfates of saturated steroids, it is probable that at least a large part of the ketosteroid conjugates not hydrolyzed by either method comprises saturated ketosteroid sulfates (such as androsterone sulfate). The possible existence of small amounts of other types of steroid conjugates (29) cannot, however, be eliminated.

Our data confirm the suggestions of others (30, 31) that hydrolysis of urine specimens with preparations of glucuronidase results in a considerable increase in the amount of assayable corticoids. This indicates that corticoid assays carried out on urines which either have been previously unhydrolyzed or have been hydrolyzed by mild acidic treatments lead to assays of only a small portion of the total corticoids in urine. Since it is improbable that corticoid sulfates are hydrolyzed by the glucuronidase concentrates, it is possible that a considerable portion of conjugated corticoids has so far escaped hydrolysis and assay. It is probable, however, that at least a portion of these corticoid sulfates is hydrolyzed in the continuous ether extraction at pH 1.0 (29) and in the buffer hydrolysis (27) procedures.

Conclusive proof that the formaldehydogenic substances released by the glucuronidase hydrolysis are indeed corticoids must await chemical isolation studies. However, the following evidence tends to support such a belief: (a) The method of urine extraction and formaldehydogenic assay is a relatively limiting procedure for corticoids. (b) The relatively large amounts of glucuronidase-released formaldehydogenic material in the urines of patients exposed to the stresses of surgery and of pregnancy also tend
to indicate that substances of adrenal cortical origin are involved. (c) The very large increase in the glucuronidase-released formaldehydogenic substances of patient C. D. (Table II), when treated with cortisone, speaks strongly for the fact that these substances are related to the adrenal steroids. Indeed, in this latter case about one-third to one-half of the administered 17-hydroxy-11-dehydrocorticosterone was accounted for as glucuronide-bound urinary corticoids.

There is some indication that a high glucuronidase to HCl-hydrolyzable steroid ratio may be correlated with a high serum $\beta$-glucuronidase activity level, since elevated serum glucuronidases are found during pregnancy (32, 33), in patients with breast cancer on estrogen therapy (34), in patients who have recently undergone surgery, and in our cortisone-treated patient. It is not surprising that this relationship appears to be more consistent for the ketosteroids than for the corticoids in view of the empirical nature of the corticoid assays obtained after HCl hydrolysis.

Experiments are at present in progress on the quantitative determination by glucuronidase hydrolysis of pregnanediol and estrogen glucuronides in urines. While these studies are still in a preliminary stage, they indicate that considerably purer concentrates of the corresponding free steroids are obtainable following glucuronidase hydrolysis than are obtained after acid hydrolysis. Our experiments with pure sodium pregnanediol glucuronidate and with sodium estriol glucuronidate reported in this paper indicate that under the conditions of hydrolysis employed a complete hydrolysis of the former but only about a 50 per cent hydrolysis of the latter steroid glucuronide is effected by the glucuronidase preparations. Pregnancy urine specimens have released 45 to 100 per cent of the acid-hydrolyzable estrogens by glucuronidase hydrolysis. While the procedures described in this paper for the glucuronidase hydrolysis of ketosteroid and corticoid glucuronides yield optimum values, our data do not indicate whether a complete hydrolysis of these urinary-conjugated steroids is thus effected. The determination of the factors involved in the limited hydrolysis of estriol glucuronide may shed some light on this problem.

**SUMMARY**

1. The preparation of calf spleen $\beta$-glucuronidase concentrates suitable for the hydrolysis of steroid glucuronides is described.
2. A procedure for the separation of glucuronic acid from pregnanediol glucuronide is described, and its usefulness in measuring the hydrolysis of pregnanediol glucuronide demonstrated.
3. Studies have been made on the effects of pH, enzyme concentration, and incubation time on the hydrolysis of urinary-conjugated corticoids.

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*Unpublished data.*
ketosteroids, and estrogens, and of the sodium salts of pregnanediol and estriol glucuronidates.

4. The optimum pH for the hydrolysis of all the steroid glucuronides studied by the β-glucuronidase concentrate was found to be about 5.0.

5. Sodium pregnanediol glucuronidate is hydrolyzed about twenty times more rapidly than the urinary corticoid conjugates, with the glucuronides of estriol and of ketosteroids being hydrolyzed at still slower rates. No significant difference in the rate of hydrolysis of sodium estriol glucuronidate in pure form or in urine was found.

6. Procedures for the hydrolysis of the conjugated ketosteroids and corticoids in urine by the β-glucuronidase concentrates have been quantitatively determined.

7. The data obtained by the application of these procedures to a number of urine specimens are presented.

8. Possible significances in variations of the ratios of acid-hydrolyzed and glucuronidase-hydrolyzed ketosteroids and corticoids are discussed.

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