rate of albumin. The remaining protein sedimented 2.2 times more rapidly than the main component.

Discussion. A rapidly migrating component is readily observed in the electrophoretic patterns of concentrated spinal fluids equilibrated with a phosphate buffer (pH 7.5, $\Gamma/2$ .05) or a veronal buffer (pH 8.6, $\Gamma/2$ 0.1). Kabat *et al.*(1) using a phosphate-sodium chloride buffer mixture at pH 7.4 and an ionic strength of about 0.2, recorded the presence of a fast component in 3 of 40 spinal fluids. Scheid and Scheid(2) studied spinal fluids which were not sufficiently concentrated and observed the fast component in 1 of 22 cases. These studies emphasize the necessity of using concentrated fluids and suitable buffer mixtures if the rapidly migrating components of spinal fluid are to be observed or separated electrophoretically.

Component X has a greater anodic mobility than albumin over a pH range varying from 7.9 to 4.0. In contrast, rapidly migrating components observed in plasma do not behave similarly over this pH range(7). Furthermore, plasma adjusted to the protein concentration of concentrated spinal fluid and equilibrated against dilute phosphate buffer does not reveal the presence of component X. Under these conditions the albumin boundary of plasma spreads appreciably and may prevent the detection of small amounts of the fast component. From the available data, it appears justifiable to assume that components X and X_1 originate in the nervous system and are proteins typical for cerebrospinal fluid.

Summary. Electrophoretic studies of 65 cerebrospinal fluids showed the presence of a rapidly migrating protein in each case. This component migrated about 1.3 times faster than albumin and sedimented at the same rate as albumin in the ultracentrifuge. In 9 cases, 2 rapidly migrating components were observed. Under the experimental conditions used, the fast components comprise about 9% of the total spinal fluid proteins.

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Received July 30, 1951. P.S.E.B.M., 1951, 78.

Hydrolysis of Conjugates of Neutral Ketosteroids. (18955)

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The study of the metabolism of steroids is, in part at least, dependent upon the determination of the quantities and types of steroids excreted in the urine. Important information could also be obtained if it were possible to estimate the individual steroid conjugates so excreted.

The usual method of hydrolyzing the steroid conjugates by refluxing strongly acidified urine has been shown to cause alterations of at least some of the steroids(1-9). Furthermore, such treatment would be expected to hydrolyze all of the conjugates to a greater

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or lesser degree. Numerous attempts have been made to reduce the drastic nature of hot acid hydrolysis by employing simultaneous extraction(10-15) and the milder and more specific method of enzymatic hydrolysis(4, 16-27).

In a previous publication(21) in which we reported that the β-glucuronidase produced by *E. coli*(28,29) hydrolyzes a large proportion of the estrogen conjugates of human pregnancy urine, we presented data to show that this means of hydrolysis also liberates a very considerable quantity of the neutral ketosteroids from these urines as well as from the urine of normal men. Subsequently, Bitman and Cohen(23) reported the use of ox-spleen β-glucuronidase for the liberation of 17-ketosteroids in urine. The data here described were obtained from a continuation of attempts to develop mild, yet effective methods of hydrolyzing the conjugated steroids and to determine their distribution in urine.

Since neutral ketosteroids are known to be excreted in part in conjugation with sulfuric acid, it would be highly desirable to have available an enzyme capable of hydrolyzing such esters. Our bacterial glucuronidase preparations which are apparently able to split all types of β-glucuronides have no effect on phenolic or alcoholic sulfates. This is also true of spleen glucuronidase(20). Further, the phenolsulfatase present in takadiastase and Mylase P preparations which effectively hydrolyzes estrone sulfate(20) has, in our hands at least, no detectable effect on the sulfates of androsterone and dehydroepiandrosterone.

Attempts to obtain sulfatases of microbiological origin have shown these enzymes to be highly specific, but none has been found to hydrolyze the sulfates of the neutral 17-ketosteroids. We have studied 23 types of bacteria as a possible source of alcoholic sulfatase. Of these only *Proteus vulgaris* and *Pseudomonas non liquefaciens*(30) exhibited sulfatase activity. The growing cultures and the culture fluids of these 2 organisms liberated sulfate from chondroitin sulfate but did not hydrolyze the sulfates of menthol, pentaerythritol, cholic acid, androsterone and dehydroepiandrosterone. The addition of these sulfates to the culture media did not stimulate the production of sulfatase. (Unpublished results).

Experimental. In the studies described below, the method of Holtorf and Koch(31) was used for estimating the androsterone and dehydroepiandrosterone, and estrone was determined by the Kober reaction according to the modification of Salter *et al.*(32). When

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TABLE I. Hydrolysis of Pure Steroid Sulfates. % recovered in form of free steroid as measured by m-dinitrobenzene reaction for ketosteroid and Kober reaction for estrone.

	Method of hydrolysis				
Conjugate	Bitman-Cohen or Talbot, *et al.*	Autoclaving, 15 lb –1 hr pH 7.3	Ether 24 hr pH 0.7	Ether 24 hr 7.2 N HCl	HCl 15 vol %, 10 min
Dehydroepiandrosterone sulfate	90–95	90	92	98	10
Androsterone sulfate	0	0	90	95	25
Estrone sulfate	—	0	95	93	98

hydrolysis of urine or of solutions prepared from butanol extracts of urine was employed, neutral fractions were obtained by the procedure of Friedgood *et al.*(33) and the neutral ketosteroids were estimated after removing the non-ketonic fraction by Girard's reagent according to the procedure of Pincus and Pearlman(3).

Hydrolysis by dilute acid. Dobriner *et al.*(14) have reported the liberation of significant quantities of neutral steroids from urine acidified to pH 1.0 and extracted continuously with ether at room temperature. Lieberman *et al.*(13) have shown that such extraction of solutions acidified to 0.2 N HCl hydrolyzes the sulfates of cholesterol, cholestanol, and dehydroepiandrosterone to the extent of 90-93%.

Talbot *et al.*(4) recovered dehydroepiandrosterone quantitatively from its sulfate by heating for 4 hours at 100°C an aqueous solution of the conjugate buffered with acetate at pH 5.5 and containing 15% $BaCl_2$. Bitman and Cohen(34) have shown that $BaCl_2$ is not necessary for the hydrolysis of this conjugate and that such treatment does not affect the glucuronide linkage(23).

We have found(15) that the methods of Talbot *et al.* and of Bitman and Cohen effectively hydrolyze dehydroepiandrosterone sulfate but do not affect androsterone sulfate to any detectable extent. This behavior is also exhibited when the aqueous solutions of these substances at pH 7.3 are autoclaved at 120°C for 1 hour. The data in Table I show the marked difference between these 17-ketosteroid sulfates when they are subjected to these very mild hydrolytic procedures.

Continuous extraction of the aqueous solutions at pH 0.7 with ether for 24 hours, at the temperature of the boiling point of ether, satisfactorily hydrolyzed the sulfates of both androsterone and dehydroepiandrosterone as well as of estrone (Table I) as measured by the respective colorimetric methods. The glucuronides of estriol, menthol, pregnanediol and phenolphthalein were not affected by this treatment. It, therefore, appears that hydrolysis at pH 0.7 under these conditions may afford a means of distinguishing steroid sulfates from steroid glucuronides.

Hydrolysis by concentrated acid. The conventional procedure for hydrolyzing the steroid conjugates by refluxing with 10-15 volumes % of concentrated HCl for 10 minutes has been shown to be destructive(1-9). The poor recovery which resulted when the aqueous solutions of pure androsterone sulfate and dehydroepiandrosterone sulfate were refluxed with 15 volumes % HCl for 10 minutes confirms this observation (Table I). Longer periods of hydrolysis yielded even lower recoveries. However, estrone is quantitatively recovered from its sulfate by this method (Table I).

Since the steroid glucuronides are not readily hydrolyzed by acid and since the destruction of the neutral 17-ketosteroids by this treatment is probably a function of the temperature and the acid concentration, we have attempted to find a set of conditions which would minimize the destruction and yet permit satisfactory hydrolysis. Using 7.2 N HCl for hydrolysis at the temperature of boiling

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TABLE II. Hydrolysis of Conjugated Ketosteroids of Normal Male Urine. Expressed as mg of dehydroepiandrosterone per 24 hr.

Urine	Method of hydrolysis: Bitman-Cohen	Ether 24 hr pH 0.7	Glucuronidase	HCl 15 vol %, 10 min	Ether 24 hr 7.2 N HCl
1	1.9	3.4	6.5	6.7	12.2
2	3.3	5	8.9	10	14.9
3	1.2	1.3	7.5	8.3	10.4
4	1.3	2	10.3	10.7	10.6
5	1.2	2.5	4.6	5.8	7.8
6	2.2	4.3	6.8	7.8	11.6

TABLE III. Hydrolysis of Conjugated Neutral Ketosteroids of Urines of Patients with Adrenal Pathology. Expressed as mg of dehydroepiandrosterone per 24 hr.

Urine No.	Type of case	pH 0.7 ether 24 hr	Glucuronidase	HCl 15 vol %, 10 min	HCl (7.2 N) ether 24 hr
1	♀—33 yr old; adrenal hyperplasia	11.4	25.8	41	46
2	♀—1½ yr old; adrenal tumor	90	8.5	65	93
3	♀—57 yr old; adrenal tumor	90	35	92	118
4	♀—2 mo old; adrenal hyperplasia	6.6	6	16.7	25.5
6	♀—6 yr old; sexually precocious	10	8	18.4	25.5

ether (35°C), androsterone, dehydroepiandrosterone, and estrone as measured by their colorimetric values are practically quantitatively recovered from their sulfates by continuous extraction with ether for 24 hours (Table I). The estrone exhibited little or no loss of biological activity as the result of this treatment. Furthermore, hydrolysis of conjugates in urine by this procedure in all but one instance gave significantly higher ketosteroid values than were obtained by refluxing with 15 volumes % HCl (Tables II and III). However, estriol and pregnanediol were recovered to the extent of only about 60% from aqueous solutions of their glucuronides. An additional 20% of estriol was obtained by treating the bicarbonate washings of the ether phase with glucuronidase. Appreciable amounts of pregnanediol could not be recovered from the ether phase, nor were additional amounts of either steroid obtained from the aqueous phase after reducing the HCl concentration to 15 vol. % and refluxing for 10 minutes. It would thus appear that a considerable destruction of these steroids had occurred. These findings may indicate that the urinary 17-ketosteroid glucuronides also are not completely recovered under these conditions but, owing to the unavailability of pure specimens of this class of compounds, this point has not been directly studied. However, the higher yields obtained by the use of 7.2 N HCl suggest that the ketosteroid glucuronides behave differently than do those of estriol and pregnanediol under these conditions.

Hydrolysis of urinary conjugated neutral ketosteroids. A study was made of the amounts of neutral ketosteroids which were liberated from the urine of normal men and of patients with adrenal pathology by the methods described above as well as by bacterial β-glucuronidase.

The normal urines were kept in the refrigerator during collection; they were then boiled at neutral pH for 10 minutes to destroy enzymes, and after removal of the free steroids with ether, they were preserved with thymol and stored in the refrigerator at 5°C.

Enzymatic hydrolysis was performed by incubating aqueous solutions of the butanol-soluble extractives of urine prepared according to Bitman and Cohen(34) with the bacterial glucuronidase for 24 hours at 38°C and at pH 6.5. An amount of β-glucuronidase capable of liberating approximately 5000 μg of phenolphthalein from phenolphthalein glucuronide in a 4-hour assay at pH 6.2 was used for each 10 ml of urine and $CHCl_3$ was used as the preservative. Butanol extracts instead of whole urine were employed for the enzymatic hydrolysis in order to avoid concen-

trations of ammonium ion which we had found(29) to have an inhibiting effect on this β-glucuronidase and which had developed in some of the urines during storage even though they were preserved with thymol. The ketosteroid values obtained when fresh whole urines are incubated are the same as those obtained with their butanol extracts. However, upon becoming ammoniacal low values are obtained with the untreated urines while their butanol extracts give the same values as those obtained on fresh urine. We have subsequently employed $CHCl_3$ as the preservative which is much superior in this regard.

The data obtained in our study of the neutral ketosteroids of the urine of normal men are presented in Table II. It can be seen that hydrolysis by the method of Bitman and Cohen liberated a significant quantity of ketosteroid in each instance. However, continuous extraction with ether at pH 0.7 without recovering unhydrolyzed steroid glucuronides from the organic phase according to Dobriner and Lieberman(35) resulted in decidedly higher values in all but one instance. This is not surprising in view of our finding that androsterone sulfate is not hydrolyzed under the conditions of the method of Bitman and Cohen but is effectively hydrolyzed by continuous extraction with ether at pH 0.7, while dehydroepiandrosterone sulfate is hydrolyzed by both procedures. It is quite likely that other neutral ketosteroids behave similarly to androsterone and dehydroepiandrosterone in this regard and that these procedures may be of great importance in enabling one to distinguish between these two types of ketosteroid sulfates. Such a distinction may be of value in steroid metabolism studies.

Hydrolysis of these urines by bacterial β-glucuronidase resulted in the liberation of more neutral ketosteroid than was obtained by continuous ether extraction at pH 0.7. Since the latter procedure apparently does not hydrolyze glucuronides and since our glucuronidase preparations contain no demonstrable sulfatase, it is tempting to assume that hydrolysis with glucuronidase measures the ketosteroid glucuronides while continuous extraction with ether at pH 0.7 gives a measure of the ketosteroid sulfates in these urines. If such an assumption is valid then the quantity of neutral ketosteroids conjugated with glucuronic acid, in every case, exceeded that conjugated with sulfuric acid.

Somewhat higher values were obtained by refluxing the urines for 10 minutes with 15 vol. % of concentrated HCl than by treatment with glucuronidase. However, with one exception, the highest values were obtained by the continuous ether extraction of the urines acidified to 7.2 N HCl. In most instances, these values (total ketosteroid?) approximate the sum of the values obtained by continuous extraction with ether at pH 0.7 (ketosteroid sulfates?) and by glucuronidase hydrolysis (ketosteroid glucuronides?).

The quantity of conjugated ketosteroids which is extracted by ether(35) from urine acidified to 7.2 N HCl was found to be very small. The bicarbonate washings of the ether obtained by treating these normal male urines in this manner, contained only 1-4.7% of the amount of ketosteroid remaining in the ether. This was ascertained both by hydrolysis with glucuronidase and refluxing with 15 vol. % HCl. The ketosteroid extracted by the bicarbonate is apparently conjugated with glucuronic acid for acid hydrolysis following treatment with glucuronidase did not give additional ketosteroid.

Although the amount of conjugated ketosteroid extracted by the ether is small under these conditions, for greater accuracy it may be desirable to treat the bicarbonate washings with glucuronidase.

Although we had shown that androsterone and dehydroepiandrosterone, measured as 17-ketosteroid, are quantitatively recovered from their sulfates in a solution of 7.2 N HCl by continuous extraction with ether at 35°C, the possibility remained that the stability of these substances under these conditions might be altered when they are present in urine. That this is not true is indicated by the recovery by this method of 88-93% of 125 γ dehydroepiandrosterone added as the sulfate to 10 ml

35. Dobriner, K., and Lieberman, S., *Steroid Hormones* (University of Wisconsin Press, 1950), page 49-50.

portions of 5 normal male urines.

The results obtained from the study of urines of patients with adrenal pathology are shown in Table III. Urines 1-4, in the form of butanol extracts(36), were kindly supplied by Dr. Willard M. Allen of the Department of Gynecology and Obstetrics of Washington University. The diagnosis of each of these cases was found to be correct by appropriate operation. Urine 6, as fresh whole urine, was obtained by Dr. Daniel Sexton of the Department of Medicine of St. Louis University from a case of sexual precocity.

Here again, as with the normal urines, ether extraction after acidification to 7.2 N HCl and removal of the non-ketonic fraction gave neutral ketosteroid values which were higher than those obtained by the other methods of hydrolysis. It is interesting to note that in these adrenal tumor cases in which the total neutral ketosteroid excretion was very high, the fraction hydrolyzed at pH 0.7 greatly exceeded that hydrolyzed by glucuronidase. In the cases of adrenal hyperplasia the total neutral ketosteroid excretion was considerably lower while the fraction hydrolyzable by glucuronidase was relatively larger. It is obvious, of course, that a larger number of cases must be studied before it can be ascertained whether this difference in distribution of neutral ketosteroid conjugates can be used to distinguish between hyperplasia and tumors of the adrenal cortex.

In preliminary studies, we have applied the color test which, according to Allen, Hayward, and Pinto(38) is specific for dehydroepiandrosterone and certain related steroids, to the neutral ketonic fraction of the hydrolysates obtained from these pathological urines. We have confirmed the observation made by these and other investigators(19,37-43) that the urines of the patients with adrenocortical tumors contain large amounts of this ketosteroid.

In addition, we have found that none of this substance was liberated by glucuronidase while a large portion of it was obtained by continuous extraction with ether at pH 0.7. It is possible that these results are related to the presence of i-androstanolone which has been shown by Dingemanse *et al.*(9) to be converted by HCl to dehydroepiandrosterone. Unfortunately, the procedure of Bitman and Cohen was not applied to these urines. Higher values were obtained when 7.2 N HCl was used. Furthermore, the color developed with the extracts following hydrolysis at pH 0.7 and in 7.2 N HCl was much better than that obtained after refluxing with 15 vol. % of concentrated HCl, readily permitting the detection of the much smaller amounts of this ketosteroid in the urines of patients with adrenal hyperplasia.

Summary. The hydrolysis of steroid conjugates has been studied by 5 methods. Continuous ether extraction of urine made 7.2 N with respect to HCl gave values appreciably higher than those obtained by boiling with 15 vol. % HCl and these values approximated the sum of ketosteroids released by β-glucuronidase and by continuous extraction with ether of urines brought to pH 0.7 with HCl.

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