Hydrolysis of Conjugated Steroids by Bacterial Glucuronidase. (17413)

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The use of acid for liberating the steroids of urine is based on the observation that these compounds are excreted in conjugated form as evidenced by their insolubility in fat solvents and by the isolation of steroid glucuronides[1-3] and sulfates.[4-6] The evidence that such treatment causes considerable loss[7-10] has led to a consideration of enzymes for the liberation of steroids from their conjugates.

Cohen and Marrian[11] and Patterson[12] have previously reported the liberation of estrogen of pregnancy urine by bacterial action and Barker, Brooksbank, and Haslewood[13] have found that pregnanediol glucuronide, and in some experiments glucuronic acid, which presumably was liberated from the conjugate were destroyed by some strains of *Staphylococcus albus*. Preparations possessing glucuronidase activity which were obtained from mammalian tissues hydrolyze the glucuronides of estriol,[14,15] pregnanediol[7,16] and other compounds[14,17,18] and Cohen and Bates[19] have studied the hydrolysis of the sulfuric acid esters of estrogens by an enzyme, phenolsulfatase, obtained from a mold, *Aspergillus oryzae*.*

Recently, we have reported[20] the production of glucuronidase by *E. coli* cultured in the presence of menthol glucuronide and an adequate oxygen supply. By this means potent culture filtrates were obtained which may be used without further purification or concentration. The present report deals primarily with the effect of this bacterial glucuronidase on the hydrolysis of some of the urinary steroids.

Experimental Procedures. The urines, preserved with thymol, were boiled at neutral pH for 10 minutes. Single specimens were boiled immediately after voiding and 24-hour samples at the end of the collection period during which time they were kept at 5°C. This precaution was taken because of the reports that glucuronidase[21] and phenolsulfatase[22] occur in urine. Free steroids were determined in these urines and the amounts of

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* Studies on hydrolysis of combined estrogens by phenolsulfatase which were discussed at the Federation meeting[20] in Detroit, April 1949, will be presented in detail in a subsequent report.

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TABLE I.
Comparison of the Influence of Different Hydrolytic Agents on Total Estrogen Liberated from Human Pregnancy Urine.*

Method of hydrolysis						
	Total estrogen (Kober) as mg estrone per l				Total estrogen as mouse units of estriol per l	
Urine	HCl	Glucuronidase	None	Urine	HCl ×1000	Glucuronidase ×1000
1	5.38	4.88	.03	A†	109	147
2	16.27	15.00	.21	B†	155	207
3	10.5	12.3	.24	C†	120	213
4	13.87	17.25	.285	D†	320	460
5	6.75	9.75	.15	E†	176	266
6	12.0	13.3	.06			

* Obtained during the last month of pregnancy.
† Ether extracts of urines A-E were not fractionated into the phenolic and neutral fractions before assay. In the bioassay (Marrian), 20 animals per dose were used and estriol was used for the standard.[2]

estrogen and 17-ketosteroid liberated from their conjugates with glucuronidase compared with the amounts liberated by acid hydrolysis.

Acid hydrolysis was performed by refluxing the urine for 10 minutes with 15 vol. % concentrated HCl. For enzymic hydrolysis, the urines with added glucuronidase were incubated for 24 hours at 38°C at pH 6.5. All hydrolyzed and unhydrolyzed samples were adjusted to pH 3 and immediately extracted with ether. Phenolic and neutral fractions were separated according to Friedgood, *et al.*,[23] ketosteroids determined by the method of Holtorf and Koch[24] after removing the non-ketonic fraction[25] and total estrogens by Salter's[26] modification of the Kober method.

The extracts obtained from the glucuronidase hydrolyzed urine gave very much less background color with the Kober reagent (absorption at 420 mμ) and a more typical color with Zimmermann's reagent than that obtained after acid hydrolysis. Furthermore, a larger proportion of the color with m-dinitrobenzene is due to ketonic compounds reacting with Girard reagent T.

Under our experimental conditions, glucuronidase qauntitatively splits glucuronic acid from menthol glucuronide as measured by Fishman's modification[27] of Miller and Van Slyke's method,[28] and hydrolyzes 95% of estriol glucuronide. Furthermore, glucuronidase preparations tested with estrone sulfate and dehydroisoandrosterone sulfate contained neither phenolic nor alcoholic sulfatase.

Results. The data in Table I show that except for urines 1 and 2, higher values for total estrogen were obtained after hydrolysis with glucuronidase than with hydrochloric acid. In addition, the estimation is more accurate because of the lower background color after treatment with Kober's reagent. Although the lower values after acid hydrolysis may be due to incomplete hydrolysis as well as destruction, it is surprising that so large an amount of estrogen is conjugated with glucuronic acid.

With the urine of pregnant women as well as that of normal men and one case of pseudohermaphroditism, glucuronidase did not liberate as much ketosteroid as that liberated by hydrochloric acid (Table II). Undoubtedly

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TABLE II.
Comparison of Hydrochloric Acid and Glucuronidase Hydrolysis of the Ketosteroids of Human Urine.

Urine No.	Method of hydrolysis: HCl	Glucuronidase	No hydrolysis
	Normal men—mg* per 24 hr		
1	—	4.25	.488†
2	—	8.3	1.176†
3	—	7.2	.612†
4	—	4.05	.450†
5	—	4.75	.546†
6	14.4	6.7	.60†
7	11.0	5.1	.416†
8‡	26.2	14.0	.448†
	Pregnant women—mg* per l		
1§	0.92	1.2	.07
2	5.5	5.0	.18
3	5.8	5.0	.26
4	10.8	7.4	.42
5	6.3	5.6	.21
6	4.8	3.6	.16

* Expressed as dehydroisoandrosterone.
† Micro-Girard separation not made.
‡ Pseudohermaphrodite.
§ Pregnancy urines 1-6 are the same as those recorded in Table I.

ketosteroid conjugated with sulfuric acid accounts for at least a part of this difference but here again, it is surprising to find so large a proportion of the ketosteroid fraction in combination with glucuronic acid.

The quantities of free estrogens and ketosteroids recorded in Tables I and II are of interest since they were obtained from fresh urine in which enzymes, if present, had been destroyed by boiling. However, even these low values may be due to enzymic hydrolysis within the bladder.

As would be expected, bacterial glucuronidase hydrolyzed sodium pregnanediol glucuronidate resulting in a 90% recovery of pregnanediol as determined by the method described by Talbot, *et al.*[29]

Dr. Ralph A. Kinsella, Jr. of this department, in preliminary studies has found that glucuronidase markedly increases the amount of cortical steroids of the urine of normal adult men as determined by a somewhat modified method of Heard, *et al.*[30]

Summary. Bacterial glucuronidase which hydrolyzes the glucuronides of estriol, pregnanediol, menthol and phenolphthalein liberates a large proportion of estrogen, ketosteroid and corticosteroid of human urine.

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