v190, 177.

15. Fiske, C. H., and SubbaRow, Y., *ibid.*, 1925, v66, 375.

16. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric Techniques and Related Methods for Study of Tissue Metabolism*, Burgess Publishing Co., Minneapolis, 1945, p159.

17. Swanson, M. A., *J. Biol. Chem.*, 1950, v184, 647.

18. Vestling, C. S., MylRoie, A. K., Irish, U., and Grant, N. H., *ibid.*, 1950, v185, 789.

19. Cori, C. F., and Cori, G. T., *J. Biol. Chem.*, 1928, v76, 755.

20. Miller, M., Drucker, W. R., Owens, J. E., Craig, J. W., and Woodward, H., Jr., *J. Clin. Invest.*, 1952, v31, 115.

21. Chernick, S. S., and Chaikoff, I. L., *J. Biol. Chem.*, 1951, v188, 389.

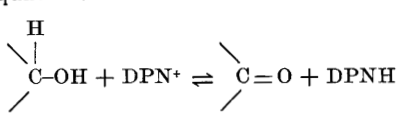
Received September 13, 1956. P.S.E.B.M., 1956, v93.

Enzymatic Estimation of Urinary Steroids.* (22819)

BARBARA HURLOCK AND PAUL TALALAY† (Introduced by Charles Huggins)

Ben May Laboratory for Cancer Research and Department of Biochemistry, University of Chicago, Ill.

Existing procedures for the determination of urinary steroids depend upon either chemical reactions or bioassay. Despite great interest in this subject and its clinical importance, simple and specific micro-methods for the estimation of the amounts and types of urinary steroids have not been perfected(1). The novel procedure to be described here permits the simple microestimation of certain types of steroids in urine. This procedure depends upon selective enzymatic oxidations and reductions of steroids and the spectrophotometric measurement of the associated changes in the concentrations of diphosphopyridine nucleotide coenzymes. Enzymatic estimations are characterized by a high degree of specificity and sensitivity. It is thus possible to obtain quantitative information on the principal types of steroid ketones and alcohols present in a few milliliters of urine without resorting to tedious isolation and separation techniques. *Pseudomonas testosteroni* is a soil bacterium capable of growing on testosterone and related steroids as its only carbon source, and of oxidizing such steroids to carbon dioxide and water(2). The presence of steroids in the growth medium of this bacterium causes the induction of certain diphosphopyridine nucleotide-linked enzymes which reversibly interconvert particular hydroxy- and ketosteroids with a high degree of steric specificity(2-4). These enzymes have been named *hydroxysteroid dehydrogenases,* and their potential usefulness for the micro-estimation of steroids was pointed out in 1952. Subsequently, steroid dehydrogenases have been applied to the measurement and identification of single steroids(2-5). The purification and substrate specificities of 2 enzymes suitable for analytical purposes have been described(5-7). Briefly stated, these enzymes are: 1) a *3α-hydroxysteroid dehydrogenase* (designated *α* enzyme) which catalyzes the reversible oxidation of 3α-hydroxysteroids of the C-19 and C-21 groups; and 2) a *3β- and 17β-hydroxysteroid dehydrogenase* (designated *β* enzyme) which catalyzes the reversible oxidations of 3β- and 17β-hydroxysteroids to their respective ketones. Thus, the specificity of these enzymes fortunately coincides with the principal types of urinary steroids *i.e.* 17-ketosteroids as well as 3α-hydroxy- and 3β-hydroxysteroids of both the C-19 and C-21 series. The *analytical procedure* depends upon interconversions of steroid alcohols and ketones according to the general equation:



* Supported by grants from Am. Cancer Society, upon recommendation of National Research Council Com. on Growth.

† Scholar in Cancer Research of Am. Cancer Soc.

when DPN^+ and DPNH refer to the oxidized and reduced forms of diphosphopyridine nucleotide respectively. DPNH has an absorption maximum ($\epsilon = 6{,}220$) at the wavelength of 340 mμ, where DPN^+ has negligible absorption. Thus, the increase in absorption at 340 mμ in the forward reaction is an accurate measure of the amount of DPNH produced and hence of the amount of steroid alcohol oxidized. Conversely, the decrease in absorption of DPNH in the *back* reaction is a measure of the amount of ketosteroid reduced. An accurate estimation of the total amounts of steroids present requires conditions under which the reaction equilibria are displaced either completely to the right (forward reaction) or to the left (back reaction), even in the presence of relatively large quantities of reaction products.

Quantitative conversions in the forward reaction were achieved by raising the pH to 9.5 and incorporating hydrazine as a ketone binding reagent into the reaction mixture. The back reaction is favored by lowering the pH, but only limited use can be made of this because of the instability of DPNH below pH 5.5. Complete reduction of ketosteroids to steroid alcohols was obtained if diphosphopyridine nucleotidase (DPN-ase) from zinc deficient *Neurospora*(9) was added to the reaction system. This enzyme destroys the DPN^+ formed during the back reaction by rupture of the nicotinamide-ribose bond of DPN^+, but does not attack DPNH.

Materials and methods. Extracts of human urine were prepared from 25 ml aliquots of 24 hour collections. Each aliquot was subjected to hydrolysis of steroid conjugates by incubation with beef liver β-glucuronidase (1000 units(10) per ml of urine) at pH 4.5 and 30° for 24 hours. Following extraction with three 15 ml portions of CH_2Cl_2, the aqueous residue was acidified to pH 1 with H_2SO_4 and further incubated at 30° for 24 hours. The residue was then reextracted with three 15 ml portions of CH_2Cl_2 and the combined solvent extracts evaporated to dryness. All evaporations were carried out in a rotating evaporator *in vacuo* below 45°. The residue was dissolved in methanol and passed over a 70 × 10 mm column of Amberlite MB 1, a strong base-strong acid, anion-cation mixed bed exchange resin obtainable from the Rohm and Haas Co., Philadelphia. The resin was well washed with water and methanol before use. The ion exchange resin quantitatively removes phenolic estrogens and eliminates other ionic constituents. The extract was evaporated, redissolved in 70% aqueous methanol and partitioned against *n*-hexane. The methanolic layer was evaporated to dryness and the residue dissolved in 0.50 ml methanol. Aliquots of this extract were then subjected to enzymatic reaction first with α enzyme and then with β enzyme in both forward and back reactions. The preparation of the enzymes has been described(7). All reactions were carried out in a Beckman DU spectrophotometer in cuvettes of 1 cm light path at approximately 25° and 340 mμ. The system for the *forward reaction* contained in a total volume of 3.0 ml: 100 μmoles of sodium pyrophosphate buffer pH 9.5, 0.5 μmole DPN^+, 1.0 millimole hydrazine sulfate (adjusted to approximately pH 9.5) and 0.025 to 0.075 ml of urine extracted in 0.1 ml CH_3OH. Initial optical density readings were taken against a control cuvette containing all ingredients except urine extract. 1000 units α enzyme in 0.01-0.02 ml were then added and readings continued until there was no further change in optical density for 15 minutes. 1000 units of β enzyme were then added and readings continued again to equilibrium. In the *back reaction,* the cuvettes contained: 100 μmoles orthophosphate buffer pH 5.5, 0.12 μmoles DPNH, 200 units DPN-ase and 0.025 to 0.050 ml of urine extract in 0.1 ml CH_3OH. Initial readings were taken at 340 mμ against a control cell containing no urine extract and no DPNH. An additional control containing only DPNH and enzymes was included, to compensate for small decreases in optical density of DPNH occurring during the course of the reaction. α and β enzymes (1000 units of each) were added successively to the reaction cuvettes and measurements made periodically until equilibrium was reached with each enzyme. The measurements were based

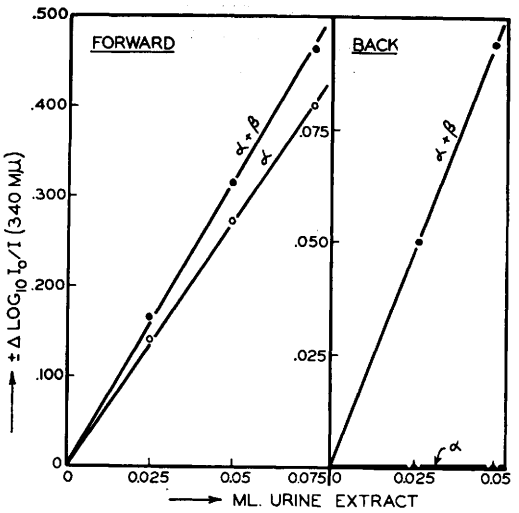


FIG. 1. Enzymatic assay of urinary steroid excretion in a 24-year-old healthy man whose output was 1350 ml in 24 hr. A 25 ml portion of urine was used to prepare 0.5 ml extract in CH_3OH as described in the text. Ordinate of the left graph shows the increases in optical density at equilibrium in the *forward reaction*, whereas that of the right graph shows the *back reaction*, in which decreases in optical density at equilibrium are plotted. Measurements were made with different amounts of urine extract and demonstrate linearity between the amount of pyridine nucleotide reduced or oxidized and vol of urine extract.

on the changes in optical density when each enzymatic reaction was complete.

Results. Typical measurements on an extract of male urine are shown in Fig. 1. Preliminary values for the steroid excretion of a small number of young subjects believed to be in good health are shown in Table I. The reliability of the assay procedure was established by several criteria: 1) A linear relation between the amount of extract and the change in optical density may be seen in Fig. 1, and has been repeatedly observed. 2) Various pure steroids added to a urine extract have been quantitatively estimated with a high degree of accuracy. The results of a recovery experiment of this type are shown in Table II. Suitable aliquots of a hydrolyzed urine extract and of solutions of purified androsterone and *epi*androsterone were each assayed successively with α and β enzymes in the *forward* reaction. The respective amounts of hydroxysteroids found with each enzyme are shown in experiments 1, 2 and 3 of Table II. A mixture of androsterone and *epi*androsterone was then assayed in the same manner and agreed within 1 percent or less with the individual assays obtained for each steroid separately (experiment 4). Similar aliquots of androsterone and *epi*androsterone were then measured in the presence of the same aliquot of urine extract and here also the recovery was better than 1% (experiment 5). Similar recovery experiments utilizing the *back* reaction have shown a recovery of *epi*androsterone measured as a 17-ketosteroid of within 1 to 2%. These experiments demonstrate the high accuracy of the method and excellent recoveries of physiological amounts of added steroids. 3) Some of the most abundant urinary steroids were added to an aliquot of urine which was then

TABLE I. Urinary Steroid Excretion in Micromoles per 24 Hours in Normal Young Adults.

Steroid groups measured	Men	Women	Examples of principal types of compounds measured
3α-Hydroxyl	54.6 (43.3 to 70.7)	34.8 (16.4 to 61.6)	Androsterone Etiocholan-3α-ol-17-one Pregnane-3α,17α,21-triol-11,20-dione Pregnane-3α,11β,17α,21-tetrol-20-one
3β-Hydroxyl + (17β-Hydroxyl)	8.86 (5.20 to 13.7)	5.98 (2.61 to 8.05)	*Epi*androsterone Dehydro*epi*androsterone
3-Ketone	<.5	<.5	Androstane-3,17-dione Etiocholane-3,17-dione
17-Ketone	21.4 (17.9 to 25.5)	9.71 (4.67 to 15.0)	Androsterone Etiocholan-3α-ol-17-one *Epi*androsterone Dehydro*epi*androsterone 4-Androstene-3,17-dione

Avg values are based on determinations in 6 men (age range 22 to 33 years) and 6 women (age range 23 to 37 years). Figures in parentheses refer to ranges of values in each group.

TABLE II. Estimation of Pure Steroids in the Presence of a Urine Extract.

Exp. No.	Material*	ml	μmoles of hydroxysteroids α	β	α + β
1	Urine extract	.05	.0580	.00725	.0653
2	Androsterone	.02	.0237	.000	.0237
3	*Epi*androsterone	.02	.000	.0435	.0435
4	Androsterone + *Epi*and'st'one	.02 .02	.0237	.0430	.0667
5	.05 ml urine extract + .02 ml androsterone + .02 ml *epi*androsterone		.0813	.0498	.131

* Urine extract was prepared from male urine as described in text (except that 1 ml extract was equivalent to 15 ml urine). Androsterone (m.p. 184.1-185.2° corr.) solution contained 3.5 mg in 10 ml methanol. *Epi*androsterone solution (m.p. 173-175° corr.) contained 6.5 mg in 10 ml methanol. Steroids were weighed to ± 0.1 mg. Values in table indicate that steroids were 94 to 96% pure by enzymatic assay.

carried through the entire procedure of hydrolysis and preparation of an extract as described. By enzymatic assay the recovery of androsterone was 94.5%, and that of pregnane-3α,17α,21-triol-11, 20-dione was 93.5%. 4) Duplicate assays on different aliquots of the same urine agreed within 5% or better.

Discussion. Several advantages characterize these enzymatic methods as compared to chemical and bioassay procedures. Sensitivity and specificity are inherent in enzymatic assays. The measurements depend upon specific changes in light absorption caused by DPNH, a compound of known structure, spectral characteristics and extinction, and may therefore be related directly to micromolar concentrations. These changes are produced by addition of a few micrograms of enzyme protein, which have negligible absorption at 340 mμ, and which produce no unspecific absorption changes or "background colors" at this or other wavelengths. Furthermore, unlike many chemical methods such as the Zimmerman reaction, equal amounts of different steroids give equivalent extinctions when measured by these enzymatic methods.

The sensitivity of the assay system depends upon the size of the reaction volume and the sensitivity of the spectrophotometer. Using microcells and a Beckman DU Spectrophotometer, 0.2 to 0.5 μg of steroid may be assayed with accuracy of a few per cent in a reaction volume of 0.2 ml. This sensitivity approaches that required for the measurement of the small amounts of steroids present in other body fluids such as the blood.

It is difficult to make comparisons between the magnitudes of the values shown in Table I and those available from other methods, because comparable measurements are available only for 17-ketosteroids. The values for 17-ketosteroids in normal young subjects by the enzymatic method are about 6 mg per day for men and 3 mg per day for women (assuming an average molecular weight of about 290). The respective ranges reported in the literature are 3 to 22 mg and 6 to 25 mg respectively(11). The enzymatic values are therefore consistent with the lower values obtained by the Zimmerman reaction, which are believed by some authors to be the most reliable(1,11).

Summary. A novel method for the sensitive and specific microestimation of steroids has been described. This method depends upon the selective oxidation or reduction of respectively hydroxy- and ketosteroids by highly purified hydroxysteroid dehydrogenases of bacterial origin. The method permits the estimation of 3α-hydroxysteroids, 3β-(and 17β-) hydroxysteroids, 3-ketosteroids and 17-ketosteroids.

The authors are indebted to Warner-Chilcott Laboratories, New York, for supplying "Ketodase" (β-glucuronidase) and to Syntex S. A. Mexico, for gifts of various steroids.

1. For review see Conference on "Methods of Steroid Determination in Blood and Urine", *Recent Progress in Hormone Research, IX,* 1954; and critical appraisal by Marrian, G. F., *Proc. of the Third Intern. Congress Biochem.,* Brussels, 1955, p511.

2. Talalay, P., Dobson, M. M., and Tapley, D. F., *Nature,* 1952, v170, 620.

3. Talalay, P., and Dobson, M. M., *J. Biol Chem.,* 1953, v205, 823.

4. Talalay, P., and Marcus, P. I., *Nature,* 1954, v173, 1189.

5. ———, *J. Biol. Chem.,* 1956, v218, 675.

6. Marcus, P. I., and Talalay, P., *Proc. Roy. Soc.*

London B, 1955, v144, 116.

7. ———, *J. Biol. Chem.,* 1956, v218, 661.

8. Barton, D. H. R., *J. Chem. Soc.,* 1953, 1027.

9. Kaplan, N. O., in Colowick, S. P. and Kaplan, N. O., eds., *Methods in Enzymology,* 1955, vII, 664, Academic Press, New York.

10. Talalay, P., Fishman, W. H., and Huggins, C., *J Biol. Chem.,* 1946, v166, 757.

11. Mason, H. L., and Engstrom, W. W., *Physiol. Revs.,* 1950, v30, 321.

Received September 13, 1956. P.S.E.B.M., 1956, v93.

Trace Metal Requirements of Azotobacter.* (22820)

RAYMOND G. ESPOSITO AND PERRY W. WILSON

Department of Bacteriology, University of Wisconsin, Madison.

Although metallic ion requirements for fixation of atmospheric nitrogen by the azotobacter have been studied for a number of years(1,2,3) an extension and reevaluation of the earlier work was thought worthwhile in light of recent advances in methodology of trace metal studies. These earlier studies established marked differences in response of both strains and species of the organism. Therefore, an added reason was to determine trace metal requirements of our strain *Azotobacter vinelandii* O which is used in many biochemical and bacteriological laboratories.

Materials and methods. *Azotobacter vinelandii* O was maintained and studied in Burk's sucrose mineral salts medium(4) in 500 ml Erlenmeyer shake flasks. The cultures were incubated at 30°C in Brunswick rotary shaker at 400 rpm. Flasks were covered with 50 ml pyrex beakers instead of cotton plugs. All glassware was cleaned by washing with detergent followed by 4 rinses in distilled water. It was then filled with 0.5% solution of tetra sodium salt of ethylene diamine tetraacetic acid (Sequestrene Na 4, Alrose Chemical Co.) and autoclaved at 15 lb. pressure for 30 minutes. After autoclaving, glassware was rinsed 5 times with ion-free water obtained by passing distilled water through a Barnstead "Bantamac" demineralizer mode Bd-1. The effluent from the column contained less than .05 ppm total ionizable solids calculated as sodium ion equivalent. The 8-OH quinoline coprecipitation technic of Nicholas(5) was used to remove traces of iron and molybdenum from growth medium. This method was not suitable for investigation of calcium, so recrystallized analytical reagent chemicals were used. Nitrogen was determined by Kjeldahl semi-micro procedure(4). Turbidities were measured with Klett-Summerson colorimeter using the 660 mμ filter.

TABLE I. Effect of Nitrogen Source on Molybdenum Requirement of *Azotobacter vinelandii* O.

	μg N_2 fixed/ml*					
Exp.	N_2 .1 ppm Mo	N_2 –Mo	N_2 .2 ppm V	NH_4^+ .1 ppm Mo	NH_4^+ –Mo	Ashed NH_4^+
I				204	186	
II	205	50		201	200	
III		50			211	86
IV		56			211	
V	212	58		279	301	88
VI	262		83			
VII	166		62			

* In all tables, the ammonia grown cells harvested and washed with water, then resuspended in water and assayed for nitrogen. In all tables, "ashed NH_4^+" was prepared by heating ammonium acetate overnight in a porcelain crucible at 150°C. Residue was dissolved in dilute acid, neutralized and added to culture flasks.

Results. In agreement with previous studies with other strains, molybdenum and iron are both specifically required for nitrogen fixation process in *Azotobacter vinelandii* O. When ammonium ion is supplied as a nitrogen source the requirement is either eliminated, as with molybdenum or spared as with iron (Tables I and II). Vanadium has been reported to replace molybdenum in certain *Azotobacter* species(6) but this does not appear to be true with strain O.

* Supported by grants from Rockefeller Foundation and Research Committee of Graduate School from funds of Wisconsin Alumni Research Foundation.