Identification of Testosterone Sulfate in Urine of Normal Adult Subjects.* (30985)

ATHANASIOS DESSYPRIS,† MICHEL A. DROSDOWSKY,† NEAL L. MCNIVEN AND RALPH I. DORFMAN†

Worcester Foundation for Experimental Biology, Shrewsbury, Mass.

Schubert and Wehrberger(1) reported the isolation of testosterone from human urine after β-glucuronidase hydrolysis. Since then, many methods have been developed for estimation of testosterone glucuronide in urine. Recently, Camacho and Migeon(2) have indicated that 0.002 to 0.139% of testosterone-4-C^{14} injected in humans was recovered as testosterone sulfate(3). This communication reports the identification and quantitation of testosterone sulfate in normal men's urine.

Apparatus. Gas chromatography was done using a model 600 Research Specialties instrument equipped with a hydrogen flame ionization detector and a solids injector. The stainless steel column 3 ft × 2 mm ID was packed with Anakrom ABS 100-110 mesh, coated with 10.5% SE-30 and operated at 225°C. The flow rate of nitrogen was 30 ml/min. Spectrophotometry was done with a model DU Beckman apparatus. A liquid scintillation Spectrometer (Packard Tricarb model 314 EX) was employed for tritium quantitation. All paper and thin-layer chromatograms were scanned to locate the testosterone zone using a Vanguard Autoscanner model 885. A Beckman IR7 infrared spectrometer was used to examine the isolated testosterone.

Samples. Several pools of male urine (3 to 7 liters) were collected and processed either immediately after collection or kept at −15°C until processing. The 24-hour urines of 3 normal males were also processed.

Methods. The free steroids (unconjugated) of the urine were extracted with three 0.6 volumes of chloroform-ether (1:3) and the solvent discarded.

After addition of NaCl to form a 20% solution the conjugates were extracted 3 times with 0.6 volumes of ethyl acetate. The combined extracts were washed with three 0.1 volumes of distilled water and the solvent discarded. The modification to the original solvolysis procedure which was suggested to us by Burstein(4) was introduced as an additional purification step and to permit a reduction of the solution volumes handled. The aqueous fraction was adjusted to pH 1 with 50% H_2SO_4. A tracer amount of testosterone-1-2-H^3 (.004 μg, S. A. 3 mc/mg) was added along with 20 g NaCl/100 ml urine. The conjugates were again extracted from the aqueous fraction 3 times with 0.6 volumes of ethyl acetate. The combined ethyl acetate fractions were kept at 37°C overnight to allow solvolysis to proceed. The solvent was washed with cold N NaOH (0.1 volume) until clear, then with distilled water until the washings were neutral. The alkaline aqueous fraction contained the unchanged glucuronide(5). The solvent was dried over Na_2SO_4 and evaporated under reduced pressure.

The extract was applied on a (0.5 or 1 mm) silica gel G TLC plate and developed by the ascending technique in the benzene-ethyl acetate (3:2) system(4). After elution of the testosterone zone, located by scanning, the extract was applied on a paper chromatogram (Whatman No. 1) and run in the petroleum ether:benzene:methanol:water (33:17:40:10) system (Bush B3). The testosterone zone was located as previously and an aliquot of the eluate (I) was assayed by gas liquid chromatography using peak heights(6).

The remaining extract was acetylated overnight with acetic anhydride in pyridine and chromatographed on paper in the ligroin-

* Supported by a grant from U.S.P.H.S. HE-01741, Am. Cancer Soc. grant P-308 and Medical Foundation of Boston.

† Present addresses: Athens Polyclinic, Athens, Greece. Laboratoire de Biochimie Medicale, Faculté de Médecine, Paris, France. Syntex Research Center, Palo Alto, Calif.

propylene glycol system. The ultraviolet absorption spectrum of the eluate (I) was determined. The acetate was hydrolyzed with 1% methanolic KOH. An aliquot of the eluate of the testosterone acetate zone was assayed by gas-liquid chromatography. The free compound was extracted with ethyl acetate, purified on a Bush B3 paper system and the infrared spectrum determined. An aliquot was incubated with a placental microsome preparation according to Finkelstein(7).

Results. Identification of testosterone. Eluate (I) on gas chromatography gave a peak having the same retention time as testosterone (RRT to cholestane 0.67). After acetylation and paper chromatography it gave on GLC the same retention time as testosterone acetate (RRT to cholestane 0.84). The ultraviolet absorption spectrum of eluate (I) showed a sharp peak at 240 mμ indicating the presence of a Δ^4-3-ketone. Hydrolysis of the acetylated material and purification by a Bush B3 paper system gave a product which had infrared bands at 1660 and 1610 cm^{-1} indicating the presence of a Δ^4-3-ketone system. However, the "fingerprint" region was too obscured by contamination to permit identification by this method.

When a portion of the last product was incubated with a placental microsome preparation(7) 17β estradiol was indicated by fluorescence measurements.

Testosterone and epitestosterone on the TLC system used had R_f values of 0.61 and 0.66 respectively and do not separate. However, on the Bush B3 system R_f values of 0.52 and 0.62 respectively were obtained. A test of the procedure using these two compounds added to urine showed that less than 4% of epitestosterone appeared in the testosterone sulfate fraction.

Quantitation. The following quantities of testosterone in the solvolysed sulfate fraction from normal male urine were found by gas chromatography.

Sample	*Sulfate Testosterone* μg/24 hr
1	6
2	7
3	10

A value of 5 μg per liter was found in a 7-liter pool of normal male urine.

Discussion. Proof for the presence of testosterone sulfate in normal male urine is based upon the following evidence: (1) Free testosterone cannot interfere since it is removed by an initial extraction. Completeness of removal is shown by the use of radioactive testosterone. (2) The solvolysis procedure hydrolyzes sulfates and not glucuronides. This is shown by the absence of radioactivity in the sulfate fraction when the procedure is applied to a child's urine to which testosterone-4-C^{14} glucuronide had been added. (3) Epitestosterone is separated from testosterone by the thin-layer and paper chromatography systems used. If present, it can amount to no more than 4% of the testosterone values. (4) The placental enzyme could also aromatize the urinary compounds androst-4-ene-3,17-dione and dehydroepiandrosterone. However, the first would be removed in the initial extraction and the latter was shown to be absent in the final extract by a negative Zimmerman test. (5) The presence of an α,β unsaturated ketone group in the final extract as shown by ultraviolet and infrared absorption indicates the presence of testosterone. The values obtained agree with the proportion of radioactivity found in the sulfate fraction by Camacho and Migeon(2).

However, the low level of testosterone sulfate is surprising in view of the ease of sulfation of testosterone by adrenal extracts as shown by Adams(8).

Summary. Testosterone sulfate was identified and quantitated in normal adult male urine. The method used involved a selective hydrolysis, thin-layer and paper chromatography followed by quantitation by gas chromatography. Values obtained were in the range of 5 to 10 micrograms per 24 hours.

We thank Dr. H. Hadd for supplying the radioactive testosterone glucuronide and D. Parsons for technical cooperation.

1. Schubert, K., Wehrberger, K., Naturwissen., 1960, v47, p281.

2. Camacho, A. M., Migeon, C. J., J. Clin. Invest., 1964, v43, p1083.

3. ———, J. Clin. Endocrin. and Metab., 1963, v23, p301.

4. Burstein, S., Lieberman, S., J. Biol. Chem., 1958, v233, 331.

5. Baulieu, E. E., Michaud, G., Corpechot, C., Ann. Biol. Clin., 1961, v3-4, p1.

6. Futterweit, W., McNiven, N. L., Narcus, L., Lantos, L., Drosdowsky, M., Dorfman, R. I., Steroids, 1963, v1, 628.

7. Finkelstein, M., Forchielli, E., Dorfman, R. I., J. Clin. Endocrin. and Metab., 1961, v21, 98.

8. Adams, J. B., Biochim. Biophys. Acta, 1963, v82, 577.

Received November 22, 1965. P.S.E.B.M., 1966, v121.

Protective Effect of Magnesium Deficiency on Experimental Allergic Encephalomyelitis in the Rat.* (30986)

PATRICIA A. MCCREARY, HECTOR A. BATTIFORA, GRANT H. LAING, AND GEORGE M. HASS

Departments of Pathology and Medicine, Presbyterian-St. Luke's Hospital, Chicago, Ill.

Acute magnesium deficiency in the rat is characterized in its earliest stage by a series of clinical and laboratory manifestations, such as pronounced hyperemia of the skin, leukocytosis and eosinophilia(1,2,3). Increased urinary excretion of histamine coincident with mast cell degranulation has also been described at this stage of the acute deficiency, and all the findings have been reported to be preventable by antihistaminic preparations(4, 5). This picture, which develops in 6-9 days, is suggestive of a transient hypersensitive state. If the animals are sacrificed after about 21-28 days on the magnesium deficient regime many microscopic lesions are found in various tissues. In cardiac and skeletal muscle, the lesions are characterized by focal perivascular collections of inflammatory cells which may be associated with muscle cell necrosis. This histological picture often resembles that seen in delayed hypersensitivity reactions. Because these findings are suggestive of an altered immunological state, the response of the magnesium deprived rat to an allergenic stimulus was tested. Experimental allergic encephalomyelitis (EAE), believed to represent a form of delayed hypersensitivity, was chosen for this purpose. When guinea pig spinal cord homogenates plus complete Freund's adjuvant are injected into the foot pads of rats, a predictable number of rats will develop EAE when histological grading of the lesions is used(6,7).

Materials and methods. Dietary regime: A synthetic diet containing less than 1 mg of magnesium per 100 g was used to produce magnesium deficiency. Control rats were fed a similar diet supplemented with 65 mg of magnesium per 100 g. The composition of the diets has been published(8).

Experiments: Male CD rats (Charles River Breeding Laboratories, Wilmington, Mass.) weighing between 100 and 120 g were individually housed in metal cages and had free access to their respective diets and distilled water.

The method of Levine and Wenk for production of EAE was utilized(6,7). All rats were pretreated with 0.6 ml of pertussis vaccine (Parke, Davis Co.) given intraperitoneally after dilution to 1.5 ml in normal saline solution. Four days later they were injected with 0.5 ml of a 33% homogenate of guinea pig spinal cord plus complete Freund's adjuvant intradermally into the foot pads. The number of animals used and the dietary regimes are illustrated in Table I. Two experiments were performed. They were designed so that the phase of hyperemia of the magnesium deficiency occurred at several intervals in relation to the administration of the encephalitogenic agent. In Experiment 1 most of the deficient rats were in the phase of skin hyperemia at time of the antigen injection. In Experiment 2 the period of hyperemia occurred at the end of the second week following the injection of Group C, and at the

* This study was supported by grants from the Dr. Grant H. Laing Cancer Research Fund, the Otho S. A. Sprague Memorial Institute and by USPHS Grants HE-09295, GM-129.