THE PREPARATION AND RENAL β-GLUCURONIDASE RESPONSE OF TESTOSTERONE GLUCURONIDE [1]

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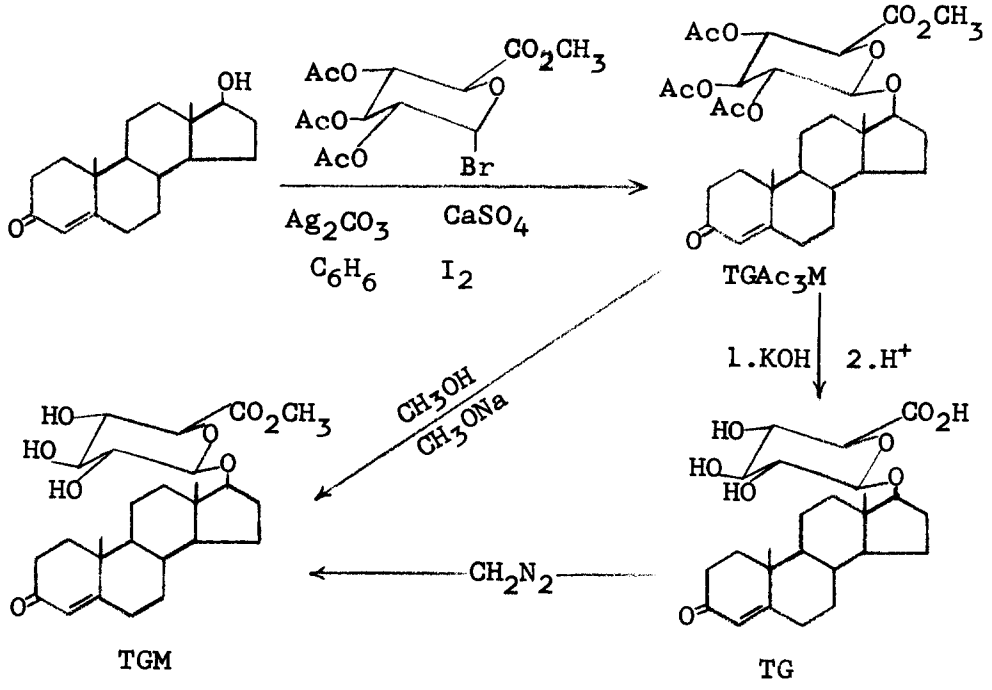
The acetal of testosterone and β-D-glucopyranosuronic acid, testosterone glucuronide (TG), has been synthesized by Wotiz et. al. by means of the Königs-Knorr reaction using methyl 2,3,4-tri-O-acetyl-1α-bromo-1-deoxy-β-D-glucuronate, followed by hydrolysis of the ester groups.[2] In order to have ample material for complete characterization and for biological testing, we have repeated its preparation. The methyl Δ^4-androsten-[(17β-1β-oside)-D-2,3,4-tri-O-acetyl-glucopyranosuronate]-3-one, $TGAc_3M$, the intermediate formed in the reaction, was separated from the excess reactants by countercurrent distribution and purified by fractional crystallization, then hydrolyzed to TG. Alternatively, $TGAc_3M$ was converted to the methyl ester of TG by transesterification. The latter was obtained also by direct esterification of TG with diazomethane.

EXPERIMENTAL

Methyl 2,3,4-Tri-O-acetyl-1α-bromo-1-deoxy-α-D-glucuronate— The action of HBr in AcOH on a mixture of methyl glucuronate tetraacetates according to Bollenbach at al.[3] gave crystals out of ethanol, m.p. 105-106°; lit. 106-107°.



Testosterone Glucuronide Triacetate Methyl Ester——A mixture of 8.80 g. (30.6mM) of testosterone, 50 g. of powdered Drierite previously dried at 250°, 57 g. of Ag_2CO_3 prepared according to Wolfrom et al.[4] and dried under vacuum, 500 ml. of freshly distilled benzene and a crystal of I_2 [5] was stirred for one hour. A solution of 12.5 g. (31.5 mM, 3% excess) of methyl 2,3,4-tri-O-acetyl-1α-bromo-1-deoxy-α-D-glucuronate in 150 ml. of benzene was added dropwise over a period of two hours to the stirred mixture. The temperature of the flask contents rose to 37°. Stirring was continued another 42.5 hr. during which aliquots were removed periodically. They were chromatographed on paper in cyclohexane/propylene glycol for 17 hr. The UV-absorbing bands (testosterone and $TGAc_3M$) were eluted from the paper with methanol, and the absorbance at 242 mμ of these solutions was measured. The % conversion ranged from 26.8 to 27.4 for all aliquots, indicating that no further reaction took place after addition of the bromo sugar was complete.

The reaction mixture was filtered, the residue washed with benzene and the combined filtrates evaporated in vacuo. The residue was acetylated with 500 ml. each of pyridine and acetic anhydride for 24 hours at room temperature. The solution was evaporated in vacuo and the residue subjected to countercurrent distribution to n=400 in ethyl acetate 50, cyclohexane 50, ethanol 60, water 40.[6,7] Aliquots of the upper phase of every 4th tube were analyzed for glucuronic acid[8] and for the Δ^4-3-keto chromaphore (Fig. 1).

The contents of tubes 182-250 (except for tube 212) were combined and evaporated. Recrystallization of the res-

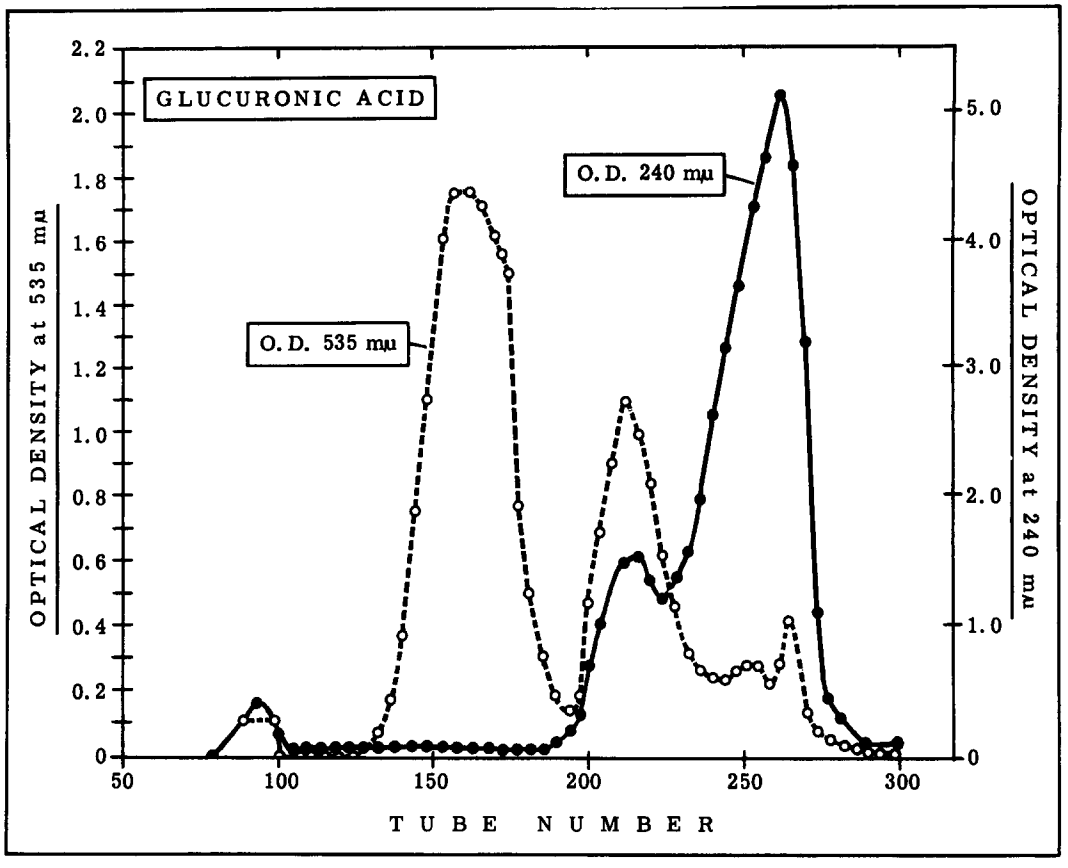


Figure 1 Countercurrent distribution of Königs-Knorr product after total acetylation. Solvent system: ethyl acetate 50, cyclohexane 50, ethanol 60, water 40. N=400 Analyses: 0.05 ml. of every 4th upper phase for glucuronic acid[8] and 0.05 ml. for optical density at 240 mμ for testosterone.

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idue in 200 ml. of MeOH gave two crops, 2.630 g. m. at 182-186° and 0.639 g. m. at 184.5-187°. Recrystallization of the first crop in 25 ml. of EtOH gave three fractions: 1.750 g. m. at 186.8-188.8°; 0.719 g. m. at 185.7-188.7°; 0.135 g.; lit. m.p. 188.2-188.5°.[2] These and the second crop (above) plus 59 mg. from tube 212 totaled 3.302 g. 5.45 mM, 17.8%.

Testosterone Glucuronide—Aqueous KOH (1.0 ml., 11.5 mM) was added to a solution of testosterone glucuronide triacetate methyl ester (1.059 g., 1.744 mM) in 100 ml. of MeOH and 50 ml. of H_2O stirred and previously chilled in an ice-salt bath. After stirring for 4 hr., the mixture was warmed to room temperature and titrated to pH 3.5 (glass electrode) with 1 N HCl: calculated, 7.0 m.e.; observed, 7.0 (4 carboxyl groups per mole). The solvent was evaporated in vacuo. Countercurrent distribution of the residue in ethyl acetate

85, n-hexane 15, water 90, acetic acid 10 [9] was carried out to n=500. Analysis of the upper phase of every 4th tube for glucuronic acid gave the curve in Fig. 2. The contents of tubes 163-250 were combined and evaporated to dryness in vacuo. The residue was dissolved in EtOH and the solution filtered and evaporated leaving a crystalline mass. A MeOH-H_2O solution of this residue was concentrated to turbidity with a stream of air, then stirred until the first crop of testosterone glucuronide was deposited, 235 mg., m.p. 174-179°; lit. 182-183.5°.[2] Two subsequent crops were obtained, 189 mg. m. at 177-181° and 231 mg.

Testosterone Glucuronide Methyl Ester——From the countercurrent distribution of $TGAc_3M$, tube 212 was examined separately. Evaporation of the solvents and two recrystallizations of the residue in MeOH gave 59 mg. of $TGAc_3M$ m. at 186.5-187.7°. It was deacylated in 5 ml. of MeOH containing 4 mg. of NaOMe at 37° for two hours. The MeOH was evaporated and the residue taken up in 5 ml. of H_2O containing 5 drops of AcOH. Extraction of the aqueous phase with $CHCl_3$ and evaporation of the solvent gave 20 mg. of crystals, m.p. 226-230°. Two recrystallizations from EtOH gave 13 mg. of TGM, m.p. 230.5-233.5°.

It was synthesized alternatively from testosterone glucuronide and diazomethane. The product, without purification, m. at 234-238°, and had an infrared spectrum identical with that prepared by transesterification.

Anal. Calcd. for $C_{26}H_{38}O_8$: C, 65.25; H, 8.00. Found: C, 65.13, 65.01; H, 7.68, 7.87.

Bioassay——The kidney β-glucuronidase assay of Fishman[10] was carried out on male AJAX mice (18-22 g.). A solution of testosterone in peanut oil was prepared by concentrating a solution of 155 mg. of the steroid and 25 ml. of Planter's Peanut Oil in 50 ml. of ether with a stream of air until the ether was removed. A homogenous suspension of TG in oil was prepared from 75 mg. of TG and 7.5 ml. of the peanut oil, ground in a homogenizer tube fitted with a motor driven Teflon pestle. In one experiment, 10 control mice received by daily injection 0.1 ml. of peanut oil, 16 received 0.62 mg. of testosterone (in 0.1 ml. of oil) and 24 received 1.0 mg. of TG (also in 0.1 ml. of oil). On each of the first four, sixth and eighth days, 1 control, 2 testosterone and 3 TG mice were sacrificed by cervical dislocation, the pairs of kidneys quickly excised, weighed and homogenized in 10 ml. of cold 0.1 M acetate buffer pH 4.5. The homogenates were diluted to 10 ml., from which 2 ml. aliquots were taken for blank and duplicate determinations. After the third day, greater dilutions were made on the kidneys of testosterone-injected mice. Enzyme actively is measured in terms of the hydrolysis of phenolphthalein mono-β-glucosiduronic acid, the released phenolphthalein being determined spectrophotometrically. The results are shown in Fig. 3. In the

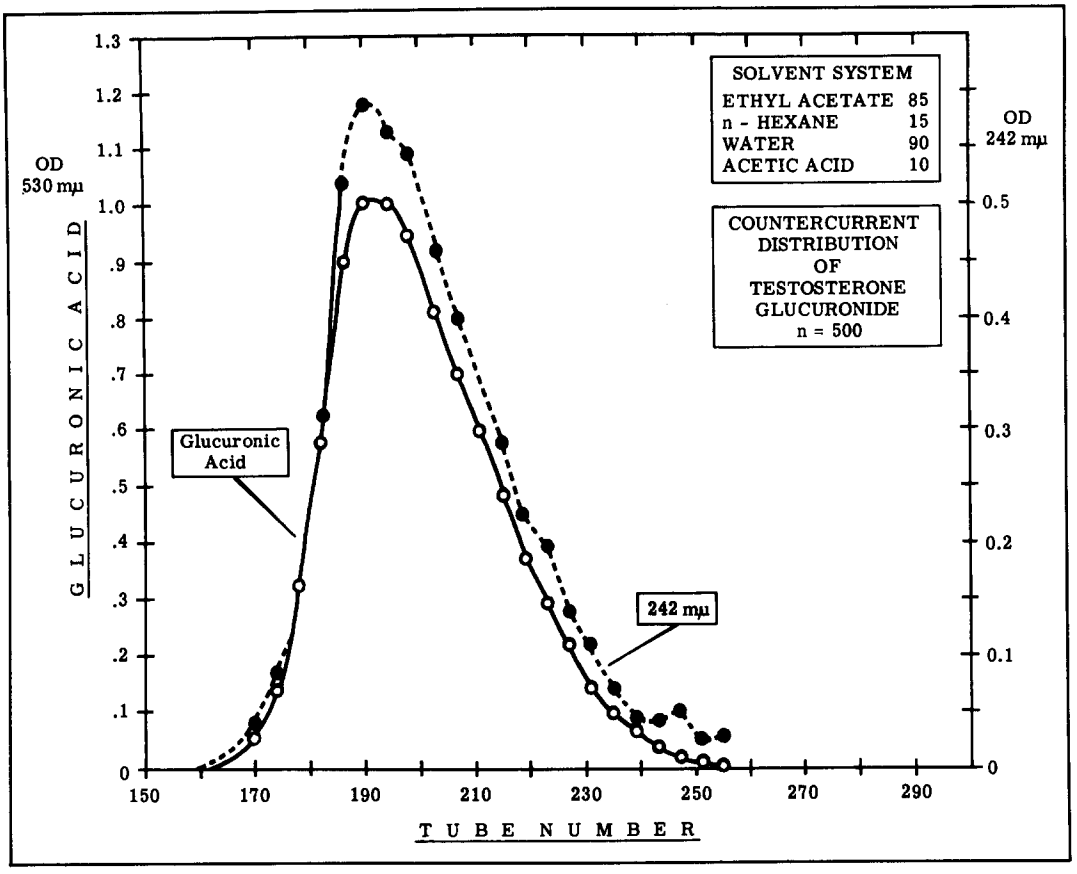

Figure 2 Countercurrent distribution of testosterone glucuronide.

second experiment (Fig. 4), 17 mice received water daily, 17 received 3.0 mg. of testosterone as an aqueous suspension, and 17 received 4.2 mg. of TG in aqueous solution. On the first, third, fifth and seventh days kidneys from 3 mice of each group were analyzed, 5 on the eighth day.

DISCUSSION

Although previous workers have customarily used long reaction times (7 to 48 hrs.) for the Königs-Knorr reaction, it is our experience with testosterone that no further reaction takes place after addition of the bromo glucuronate is complete.[11] Acetylation of the reaction mixture simplifies its purification by converting unreacted bromo glucu-

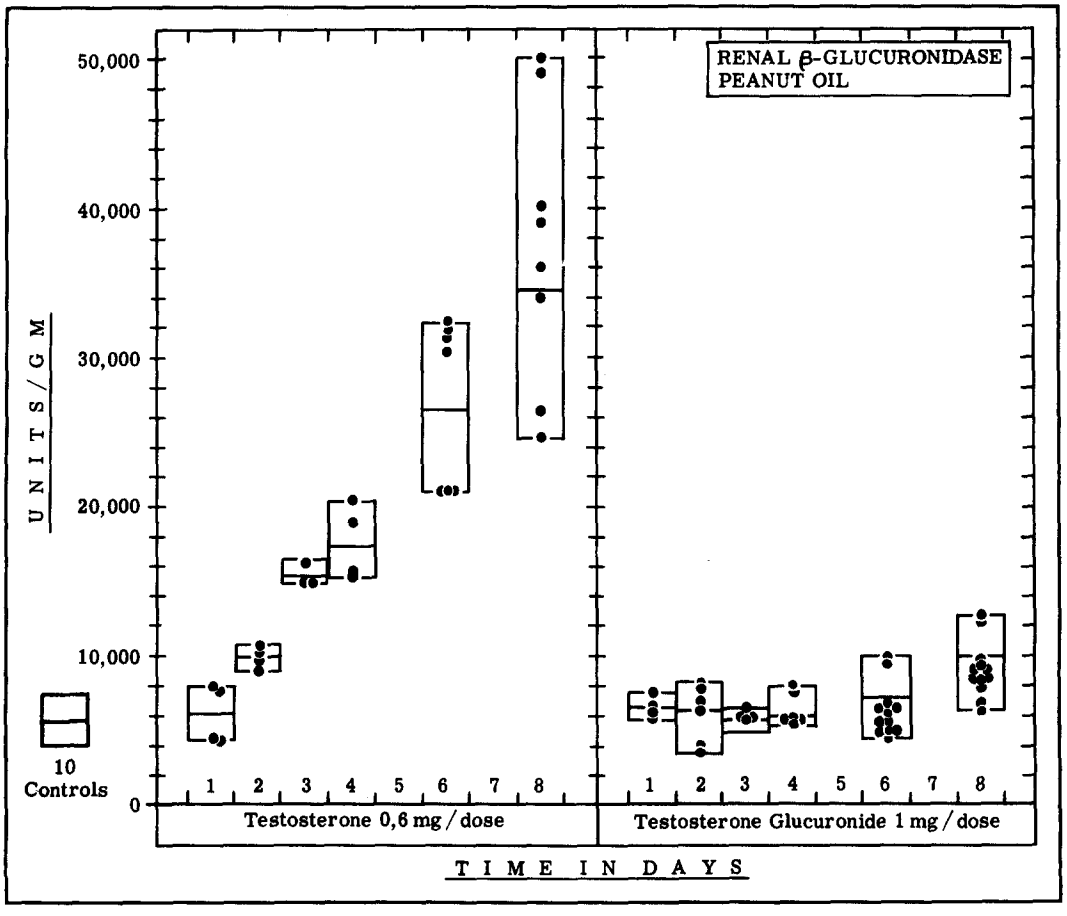

Figure 3 Renal β-glucuronidase assay of TG and testosterone in peanut oil.

ronate to the anomeric methyl glucuronate tetracetate and unreacted testosterone to testosterone acetate. It is believed that the latter is better separated from $TGAc_3M$ in the countercurrent distribution (Fig. 1) than testosterone would be, and that this separation accounts for the ease of crystallization of $TGAc_3M$. The yield of 17.8% of pure products may be compared with Wotiz' 29% yield of material of unstated purity. It is interesting that their product contained a molecule of ethanol when crystallized out of 50% ethanol, whereas our product out of either ethanol or me-

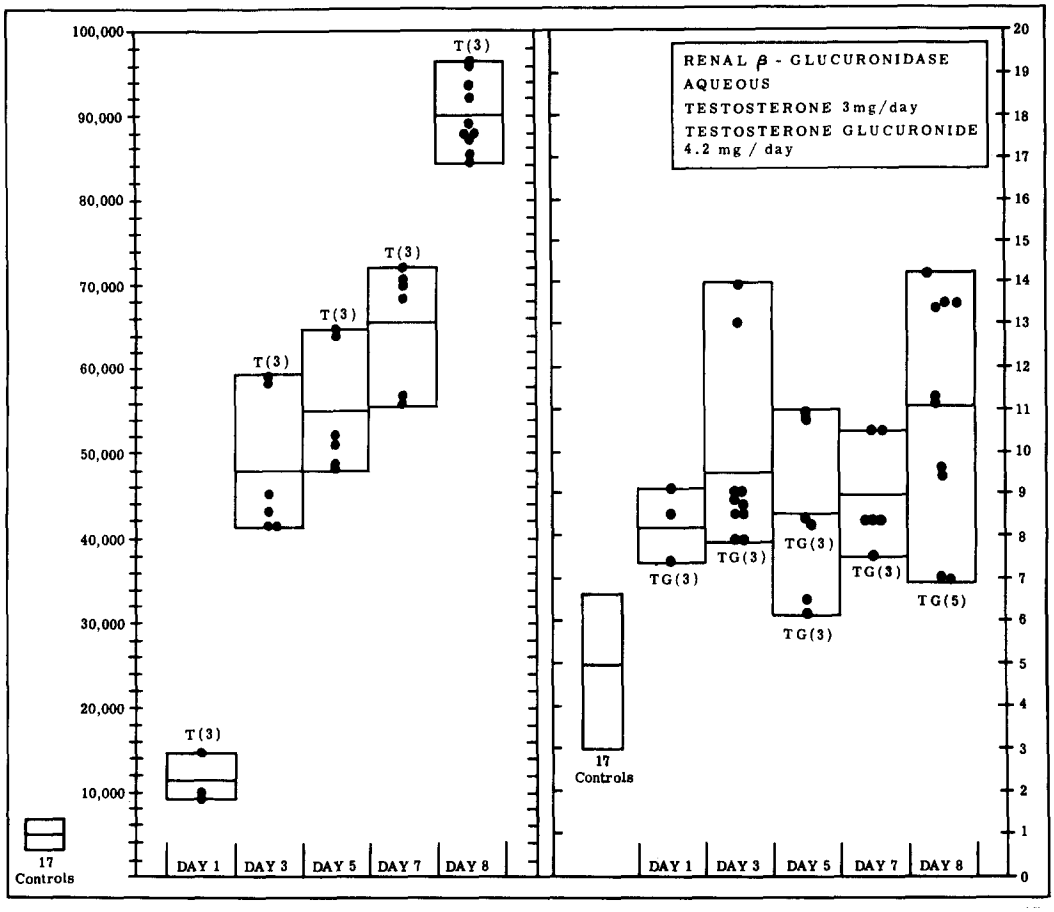

Figure 4 Renal β-glucuronidase assay of TG and testosterone in water.

thanol was unsolvated.

Alkaline hydrolysis gave testosterone glucuronide which, even after purification by countercurrent distribution in which it appeared to be substantially a single component (Fig. 2), was difficult to crystallize. Base catalyzed methanolysis gave testosterone glucuronide methyl ester, which was identified by its elemental analysis, infrared spectrum and synthesis from TG and diazomethane.

The infrared spectrum of testosterone glucuronide triacetate methyl ester in chloroform is nearly identical with

that determined on the melt by Smakula et al.[12] Their curve on a nujol mull of the sample differs primarily in the presence of a 2.81 μ band indicating the ethanol of crystallization that is lost when the sample is melted. Hydroxyl bands near 3 μ in the curve obtained on a mulled sample of testosterone glucuronide methyl ester indicate strong hydrogen bonding. Bands at 10.94 and 11.47 μ clearly identify the β-glucosidic linkage, also are present in $TGAc_3M$ and TG spectra, though not so well defined. The ester carbonyl band at 5.74 μ is replaced by a broad carboxyl band at 6 μ in the curve of testosterone glucuronide, overlapping the conjugated ketone carbonyl band. The NMR spectrum of $TGAc_3M$ was determined in $CDCl_3$.[13] The ORD curves of TG and $TGAc_3M$ demonstrate a negative multiple Cotton effect and are very similar to that of testosterone.[14]

The β-glucuronidase assay of Fishman is based on his observation that the level of this enzyme in the AJAX mouse kidney is greatly increased on administration of testosterone. As shown in Fig. 3, 0.6 mg. of testosterone per day suspended in peanut oil raised the level of enzyme from 5,000 units per g. of kidney to 34,000 units per g. in eight days. During that period the administration of 8 mg. of TG raised the level only to 10,000 units per g., a level reached with 1.2 mg. of testosterone. Since 8 mg. of TG is equivalent to 6 mg. of testosterone, TG is one-fifth as active in this assay as testosterone. In aqueous solution, 33.6 mg. of TG (in 8 days) produced a level of 11,000 units, a level

reached in one day with 3 mg. of testosterone. TG is thus one-eighth as active as testosterone in this medium.[15]

Two lines of evidence suggest that the renotropic activity of TG is not merely the result of free testosterone from the partial hydrolysis of TG. The stability of a glucuronide toward hydrolysis was shown by Siiteri and Lieberman's demonstration that ^{3}H-androsterone glucuronide was excreted unchanged.[16] TG is metabolically active, but its array of metabolites is different from that of testosterone.[17] Thus, TG may be an important member of the androgen community. In a normal male its concentration in the plasma is four times that of free testosterone.[18] Our renotropic assay indicates TG is about one-eighth to one-fifth as active as testosterone, consequently 33 to 44% of the circulating androgen could be accounted for as TG.

As stated above, TG probably does not undergo partial hydrolysis in or prior to the kidney; it obviously is not completely hydrolyzed or its β-glucuronidase response would have paralleled that of testosterone. Thus, the simultaneous conditions of TG, enzyme and low pH are not met in the kidney. No information is available concerning the relative rates of metabolism of TG and testosterone, nor of their kidney clearance mechanisms, but tubular secretion apparently plays a prominent role in the clearance of glucuronides of certain corticosteroids.[19]

The recent isolation and identification of TG from human blood[20] further emphasizes its importance in androgen metabolism.

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