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5-Pregnene- 3β ,20 α -diol-3-sulfate-20-(2'-acetamido-2'-deoxy- α -D-glucoside) and 5-Pregnene- 3β ,20 α -diol-3,20-disulfate. Two Novel Urinary Conjugates*

Martha Arcos and Seymour Lieberman

ABSTRACT: Two new crystalline conjugates have been isolated from the urine of normal human subjects to whom pregnenolone was administered. One of the compounds was the 5-pregnene- 3β ,20 α -diol-20-(2'acetamido-2'-deoxy- α -D-glucoside). Its structure was proved by elemental analysis, infrared spectra, nuclear magnetic resonance (nmr) spectra, and the products formed by hydrolysis. Mild acid hydrolysis yielded 5-pregnene- 3β ,20 α -diol and *N*-acetyl-D-glucosamine. Treatment with commercial beef glucuronidase yielded the known pregnenediol-3-monosulfate, thus locating

In an attempt to evaluate the influence that the secretion of pregnenolone and its sulfate would have upon the estimation of the secretory rate of progesterone, experiments were performed in which tracer doses of 3H-labeled pregnenolone were administered intravenously to normal subjects (Arcos et al., 1964). The experimental design required that the urinary metabolites be isolated as conjugates so that the sulfates could ultimately be separated from the glucuronides. For this purpose, the mixture of urinary conjugates was chromatographed on Celite using systems which previously (Siiteri et al., 1963) had been shown capable of effecting this separation. In the course of these analyses, a 3H-labeled conjugate was found in fractions of the chromatogram which were eluted much later than those containing the monoglucuronides. (The monosulfates are usually found in these chromatograms in fractions preceding those containing the monoglucuronides.) Our interest in this polar material was further aroused when preliminary experiments revealed that the steroid moiety of this conjugate could not be

both conjugating groups. Solvolysis gave the desulfated *N*-acetylhexosaminide from whose optical rotatory dispersion (ORD) curve the α configuration of the glycosidic bond was established. Thus, for the first time, a steroid metabolite conjugated with *N*-acetylglucosamine has been isolated from human urine. The results show that cleavage by crude glucuronidase preparations cannot itself offer proof of structure of conjugates. The second new metabolite of pregnenolone was the disulfate of pregnenediol. Of the pregnenolone produced *in vivo*, 8% is excreted as these two conjugates.

liberated in free form by either Ketodase hydrolysis or by solvolysis. The following results will serve as an illustration. Subsequent to the intravenous administration of a tracer dose of 3H-labeled pregnenolone, a polar fraction representing 8% of the injected tritium was obtained by chromatography. Attempts to solvolyze this fraction (with HClO₄ in tetrahydrofuran) liberated only 8% of the 3H as ether-soluble material. The aqueous-soluble conjugated fraction was then treated with Ketodase (incubation for 4 days) but this produced only an additional 28% of the radioactivity. Finally, the remaining conjugated material was hydrolyzed by boiling with HCl and this process converted about 40%of the radioactivity originally present into an ethersoluble form. Although this conjugate is not a glucuronide (vide infra), it is partially hydrolyzed by the commercial preparation of β -glucuronidase (Ketodase) and thus, it was evident that the bulk of the radioactivity found in this polar fraction (8% of the injected dose) was present as a conjugate possessing unusual hydrolytic properties. The present paper describes the isolation of this substance and the experiments which lead to the assignment of its structure as 5-pregnene- 3β ,20 α -diol-3-sulfate-20-(2'-acetamido-2'-deoxy- α -Dglucoside) (II) (Figure 1).

Since pregnenolone is probably not produced by normal individuals in amounts that exceed a few

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^{*} From the Departments of Biochemistry and of Obstetrics and Gynecology, College of Physicians and Surgeons of Columbia University, New York, New York. *Received February 28, 1967*. This research was supported by Grants AM-00110 and TIHD13 of the U. S. Public Health Service.



FIGURE 1: 5-Pregnene- 3β ,20 α -diol-3-sulfate-20-(2'-acet-amido-2'-deoxy- α -D-glucoside) (II).

milligrams a day, the weight of the polar conjugate ordinarily excreted in urine could hardly be expected to be sufficient for structural studies. In fact, Wilson and Lipsett (1961) had already shown that, probably its most abundant urinary metabolite, 5-pregnene-3 β - 20α -diol, is excreted in about 0.5-mg amounts/day. Consequently, in order to improve the chances of isolating the polar conjugate in satisfactory quantities, unlabeled pregnenolone was fed to several normal subjects. To one, a tracer dose of pregnenolone- 7α -³H was also injected intravenously. In this way, a pool of urine containing milligram amounts of 3H-labeled conjugates was obtained. After elaborate chromatographic analysis, two conjugates were isolated from the polar fractions in crystalline form. One was shown to be 5-pregnene- 3β , 20α -diol-3, 20-disulfate (I) by mixture melting point with an authentic sample and by its infrared spectrum (Figure 2).

The second conjugate was shown to be a sulfate by virtue of a positive methylene blue test and by the presence of a wide absorption band between 1235 and 1215 cm⁻¹ in its infrared spectrum. The most revealing aspect of its infrared spectrum was the presence of two absorption bands at 1635 and 1550 cm⁻¹, which are usually associated with the amide I and amide II bands, respectively (Bellamy, 1958). The conjugate failed to give the naphthoresorcinol test for glucuronides. When hydrolyzed with concentrated HCl it gave a positive, albeit weak, reaction with *p*-dimethylaminobenzaldehyde (Suzuki and Strominger, 1960), a characteristic test for N-acetylamino sugars. That II was a conjugate of an N-acetylhexosamine received further support from its nuclear magnetic resonance (nmr) spectrum, which contained a resonance peak at τ 7.65 arising from the proton on the nitrogen atom of the acetamido group. A larger peak, at τ 7.15 was associated with the four protons present in the ammonium ion of the sulfate.

Mild acid hydrolysis with dilute HCl permitted the identification of both the steroid and the amino sugar moieties. The steroid aglycone proved to be 5-pregnene- 3β ,20 α -diol as shown by its R_F in the Bush B2 paper chromatographic system (Bush, 1952) and by a reverse



1800 1700 1600 1500 1400 1300 1200 1100 1000 900 800 700 Frequency (cm⁻¹).

FIGURE 2: Infrared spectra determined in KBr. (A) Ammonium 5-pregnene- 3β ,20 α -diol-3-sulfate-20-(2'-acetamido-2'-deoxy- α -D-glucoside) (II); (B) 5-pregnene- 3β ,20 α -diol-20-(2'-acetamido-2'-deoxy- α -D-glucoside) (III); (C) diammonium 5-pregnene- 3β ,20 α -diol-3,20-disulfate (I).

isotope procedure. The hexosamine was shown to be *N*-acetyl-D-glucosamine by its melting point and infrared spectrum.

The location of the two conjugating groups was established in the following way. As mentioned before, incubation with Ketodase resulted in cleavage of the hexosaminidic link and isolation of the known 3-monosulfate of 5-pregnene- 3β ,20 α -diol. Identification of this sulfate, which had already been isolated from urine by Calvin and Lieberman (1966), was established by its melting point, infrared spectrum, and oxidation to pregnenolone sulfate. These results demonstrated that the sulfate group was located at C-3 and the *N*-acetylglucosamine residue was at C-20. It also added further support for the identity of the steroid moiety.

Solvolysis of the conjugate removed the sulfate group and left the crystalline *N*-acetylhexosaminide (III). In accord with the assigned structure were the elemental analysis and the infrared spectrum which retained the two amide absorption bands at 1635 and 1550 cm⁻¹, present in both II and *N*-acetylglucosamine.

In order to ensure that the hydroxyl groups of the hexosamine residue present in II were unsubstituted, II was acetylated with acetic-³H anhydride of high specific activity. Following removal of the sulfate group by solvolysis, the isotope content of the product confirmed the presence of three acetylatable hydroxyl groups. Elemental analysis of the crystalline triacetate was consistent with this formulation. Optical rotatory dispersion (ORD) measurements made on the solvolyzed product (III) proved that the hexosaminidic bond was α .

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FIGURE 3: Outline of procedure used for the isolation of the two conjugates. (*) Capital letters refer to Celite partition chromatography systems (Table I). (†) Numbers in brackets refer to the holdback volumes in which the peaks appeared.

Experimental Section

The following chemicals were purchased from commercial sources: 5-pregnenolone- 7α -³H and progesterone-4-14C from New England Nuclear Co. (purified as previously described (Arcos et al., 1964)); unlabeled 5-pregnenolone, N-acetylglucosamine, N-acetylgalactosamine, and N-acetylmannosamine from Mann Research Laboratories, Inc; 5-pregnene- 3β , 20α -diol from Steraloids; and Ketodase (β -glucuronidase) from Warner-Chilcott Laboratories. All solvents were of analytical grade or were distilled before use. Melting points were determined on a Kofler block and are corrected. Infrared spectra were obtained using a Perkin-Elmer Model 221 spectrometer. Radioactivity was measured, as previously described (Arcos et al., 1964), using a Packard Tri-Carb liquid scintillation spectrometer, Model 314-DC. ORD measurements were made using a Bendix-Ericcsen automatic recording spectropolarimeter, Model Polarmatic 62. The Celite (Johns Manville, No. 545), used for column partition chromatography, was washed and the columns were packed by the methods described by Siiteri (1963).

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only one typical procedure for the collection of urine and the extraction and isolation of the steroid conjugates will be described. Each of eight normal subjects ingested 1.5 g of unlabeled 5-pregnenolone (0.5 g/day). Their urine was collected for 5 days, pooled, and stored in the cold room until further treatment. Another normal subject was injected intravenously with 10 μ c of 5-pregnenolone- 7α -³H and 1 μ c of progesterone-4-¹⁴C; at the same time he ingested unlabeled 5-pregnenolone in the same dosage as above. His urine, which was also collected for 5 days, was added to the pool. In this way, a urinary extract was obtained which contained milligram amounts of the ³H-labeled polar conjugates.

The pooled urine (47.5 l.) was diluted with an equal volume of water after which the pH was adjusted to 5–6, using a solution of 50% sulfuric acid (Arcos and Lieberman, 1965). Charcoal (Darco 61) (380 g) and an equal weight of Celite were added and the suspension was stirred mechanically at room temperature for 2 hr. The charcoal and Celite were removed by filtration on a Buchner funnel (32-cm diameter). The cake was then transferred to a stainless-steel beaker and was leached by stirring with 4 l. of a mixture of ethyl

alcohol-1 M ammonium hydroxide (9:1). The leaching process was repeated twice more. The spent urine was adsorbed a second time with charcoal and the pooled ammoniacal ethanol from both extractions was evaporated to dryness, leaving 137 g of a brown, viscous residue. This was redissolved in 700 ml of water and the solution was extracted three times with 500-ml portions of *n*-butyl alcohol. This distribution effected an important purification step since evaporation of the organic solvent to dryness left a brown residue weighing only 42 g. The conjugates were then obtained from this crude mixture by repeated column partition chromatography (Figure 3).

The 42-g residue was separated into fractions containing the monosulfates and monoglucuronides labeled with both ³H and ¹⁴C and those having the polar conjugates labeled only with ³H (Arcos *et al.*, 1964), using system A (Table I) on 600 g of Celite, holdback

TABLE I: Chromatography Systems.

- A Isooctane (20)-*t*-butyl alcohol (50)-1 м ammonium hydroxide (50)
- B Isooctane (17.5)-*t*-butyl alcohol (50)-1 M ammonium hydroxide (50)
- C Isooctane (4)-chloroform (2)-*n*-butyl alcohol (2.5)-methyl alcohol (2)-0.3 м pyridinium sulfate (3)-pyridine 5% of the stationary phase.
- D *n*-Butyl alcohol (50)-glacial acetic acid (12)water (25)
- E Chloroform (95)–ethyl alcohol (5)
- F Isooctane (10)-*t*-butyl alcohol (5)-water (5)
- G Isooctane (17.5)-*t*-butyl alcohol (50)-water (50)
- H Benzene (8)-isooctane (2)-methyl alcohol (8.5)water (1.5)
- I Benzene (7)-isooctane (3)-methyl alcohol (8.5)-water (1.5)

volume of 1100 ml. The radioactive eluates obtained between four and twelve holdback volumes were pooled, the solvents were evaporated, and the residue (19.5 g) was rechromatographed using the same system on 250 g of Celite, holdback volume of 450 ml. The material (3.5 g) obtained from the fractions eluted between 5 and 13 holdback volumes, was rechromatographed using system C on 150 g of Celite, holdback volume of 240 ml. The conjugates (700 mg), eluted between 5 and 14 holdback volumes, were rechromatographed using the same system on 100 g of Celite. holdback volume of 165 ml. Two ³H-labeled products were obtained: one eluted between six and ten holdback volumes, eluate A (220 mg), and the other eluted between 11 and 14 holdback volumes, eluate B (156 mg).

5-Pregnene- 3β , 20α -diol-3, 20-disulfate (I). Eluate A

was chromatographed again using system C on 45 g of Celite, holdback volume of 80 ml. 3H-labeled product (100 mg) was eluted between seven and nine holdback volumes and this was rechromatographed using system B on 45 g of Celite, holdback volume of 80 ml. The ³H-labeled material (40 mg) which was eluted between eight and eleven holdback volumes was rechromatographed using system B on 26 g of Celite, holdback volume of 45 ml. The amorphous product, eluted between eight and eleven holdback volumes, was crystallized from methyl alcohol and methylene chloride yielding 15 mg of I that melted at 198-200°. Its infrared spectrum was identical with that of a synthetic sample of the diammonium salt of 5-pregnene- 3β , 20α -diol sulfate (Figure 2). Admixture of the two compounds gave no depression in melting point.

The diammonium salt of pregnenediol disulfate was synthesized by treatment of 5-pregnene- 3β , 20α -diol with the pyridine-SO3 complex (Calvin and Lieberman, 1964). A cooled (0°) solution of 120 mg of 5-pregnenediol in 1 ml of dry pyridine, was slowly added to a solution of 0.18 ml of chlorosulfonic acid in 1 ml of dry pyridine. Immediately, the reaction mixture was heated in a water bath to dissolve the precipitate which had formed. After standing overnight at room temperature, the mixture was treated with 10 ml of 7 N ammonium hydroxide and then was extracted twice with 50-ml portions of n-butyl alcohol. The organic extract was separated and evaporated to dryness leaving a product that was purified by chromatography using system B on 45 g of Celite, holdback volume of 80 ml. The disulfate was eluted between six and nine holdback volumes, and was crystallized from methyl alcohol. The diammonium salt (75 mg) crystallized as platelets melting at 198-200°. Its infrared spectrum is shown in Figure 2.

Anal. Calcd for $C_{21}H_{40}N_2O_8S_2$: S, 12.50. Found: S, 12.28.

5-Pregnene-3\,20\alpha-diol-3-sulfate-20-(2'-acetamido-2'deoxy- α -D-glucoside) (II). Eluate B, weighing 156 mg, was rechromatographed, using system A, on 45 g of Celite, holdback volume of 80 ml. The residue (55 mg), eluted between eight and eleven holdback volumes, was recrystallized several times from methyl alcohol and methylene chloride. The conjugate II, which crystallized as long needles, begins to decompose at 189° , $[\alpha]_{D}^{25} - 19.8^{\circ}$ (c, 4.900 mg in 3 ml of methyl alcohol), $[m]_D - 134.4^\circ$. Its infrared spectrum (Figure 2) contained two absorption bands at 1635 and 1550 cm⁻¹ compatible with the amide I and II bands (Bellamy, 1958), and one wide band between 1235 and 1215 cm⁻¹ which is associated with the sulfate group. The sodium salt was prepared by dissolving 5 mg of II in a 20% solution of sodium chloride. The salt was extracted into tetrahydrofuran and, after evaporation of the solvent, was crystallized from methyl alcohol and methylene chloride. It melted at 212-214°.

Anal. Calcd for $C_{29}H_{47}NO_{10}NaS \cdot 3H_2O$: C, 51.30; H, 7.87; N, 2.06; S, 4.72. Found: C, 51.54; H, 7.73; N, 2.35; S, 4.75.

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solution of 5.2 mg of II in 4 ml of distilled water was acidified with 0.04 ml of 10 N HCl. The solution was heated, in a loosely stoppered test tube, for 90 min in a boiling water bath (Ryan et al., 1965). The free steroid was extracted with three portions of 3 ml of chloroform. The aqueous phase was evaporated to dryness and the residue was chromatographed on paper (Whatman No. 1), in parallel with samples of N-acetylglucosamine, N-acetylgalactosamine, and Nacetylmannosamine, using system D (Suzuki and Strominger, 1960) for 9 hr. The sugars were spotted by the silver nitrate procedure of Trevelyan et al. (1950). The R_F of the unknown sugar was 0.31, whereas those of the authentic samples were: N-acetylglucosamine, 0.29; N-acetylgalactosamine, 0.35; and Nacetylmannosamine, 0.39. The isolated hexosamine was eluted from the paper with methyl alcohol and crystallized from methyl alcohol and ether as dendrites, melting at 192-194°. Its melting point as well as its infrared spectrum were identical with those of Nacetyl-D-glucosamine.

The chloroform-soluble steroid, liberated by acid hydrolysis, was chromatographed on alumina containing 6% water, by stepwise elution with mixtures of ligroin in benzene and ethanol in benzene as previously described (Arcos et al., 1964). The radioactive steroid was eluted with 0.4% ethanol in benzene. One aliquot of the isolated compound was chromatographed on paper, in parallel with a sample of 5-pregnene- 3β , 20α diol, using the Bush B2 system (Bush, 1952). Only one radioactive spot was detected and it had the same R_F (0.61) as the reference pregnenediol. Another aliquot (1.5 mg) was diluted with 10 mg of unlabeled 5-pregnene-3 β ,20 α -diol and the mixture was acetylated with acetic anhydride in pyridine. The acetylated product was chromatographed on alumina containing 6% water, by stepwise elution with mixtures of ligroin in benzene. The crystalline diacetate was eluted with benzene-ligroin (4:6) and recrystallized from methyl alcohol to constant specific activity. The specific activities, expressed as counts per minute per milligram, were as follows: 429 and 395 cpm/mg for the products from the first and second crystallizations, respectively, and 405 and 406 cpm/mg for the residues left in the first and second mother liquors.

B. INCUBATION WITH KETODASE. The pH of a solution of 8.7 mg of II in 50 ml of distilled water was adjusted to 5.2 by the addition of 10 ml of sodium acetate buffer (0.2 M). Ketodase (300 units/ml) was added and the mixture was incubated at 37° for 4 days. After addition of solid ammonium sulfate (50 g/100 ml), the incubation mixture was extracted three times with one-half its volume of a mixture of ether-ethanol (3:1). Evaporation of the organic solvents left a residue which was chromatographed using system A on 30 g of Celite, holdback volume of 48 ml. Two ³H-labeled products were isolated: one eluted between two and three holdback volumes.

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The second was identified as II, the original conjugate, by its infrared spectrum. The material eluted first, crystallized from methyl alcohol and methylene chloride, and melted at 196-198°. Its infrared spectrum identified it as 5-pregnene- 3β ,20 α -diol-3-monosulfate which had previously been described by Calvin and Lieberman (1966). Its structure was further substantiated by conversion to pregnenolone via pregnenolone-3sulfate. An aliquot of the isolated 5-pregnenediol-3monosulfate was oxidized using a solution of 1%CrO₃ in dry pyridine as described by Calvin and Lieberman (1966). The oxidation product was purified by chromatography on Celite using system A. The material, eluted between two and three holdback volumes, was solvolyzed in tetrahydrofuran with HClO₄ (Burstein and Lieberman, 1958). The cleaved product was then purified by thin layer chromatography using system E in parallel with a sample of authentic pregnenolone. The product, migrating at the same rate as the authentic sample (R_F 0.45), was crystallized from methyl alcohol and melted at 190-192°. Its infrared spectrum was identical with that of pregnenolone. In another experiment where II was incubated with Ketodase, under the same condition described above, 20% was found to be hydrolyzed in 24 hr and 50% in 72 hr. These values were determined by estimating the amount of unhydrolyzed II recovered after incubation and subsequent chromatography (system A).

C. SOLVOLYSIS. To a solution of 5 mg of II in 1 ml of water and 15 ml of tetrahydrofuran, was added 0.03 ml of 70% HClO₄. The mixture was heated for 1 hr at 50° and then was left at room temperature overnight. After neutralization and evaporation of the solvents, the residue was chromatographed using system F on 26 g of Celite, holdback volume of 45 ml. One radioactive peak was obtained in the second holdback volume. The product, so obtained, was recrystallized several times from a mixture of ether and methanol containing 1 or 2 drops of water. It formed rosettes melting at 268–272° (III), $[\alpha]_D^{25} - 17.0°$ (c, 3.000 mg in 3 ml of methyl alcohol, $[m]_D - 94.8°$.

Anal. Calcd for $C_{29}H_{47}NO_7 \cdot 2H_2O$: C, 62.45; H, 9.21; N, 2.51. Found: C, 62.68; H, 9.68; N, 2.66.

The infrared spectrum of this compound exhibited the absorption bands at 1635 and 1550 cm⁻¹ (Figure 2) characteristic of the amide I and II bands. Compound III could also be obtained by solvolysis in dioxane without the use of mineral acid. Storing II overnight in dioxane containing 5% water, followed by heating for 5 min in a boiling water bath, was sufficient to cleave the sulfate quantitatively yielding III in an easily purified form.

Acetylation. To 20 mg of II, suspended in 0.5 ml of dry pyridine, 1.5 ml of acetic anhydride was added. The reaction mixture was kept in a water bath at 50° for 4 hr, during which time the compound dissolved. After storing the solution overnight at room temperature, methyl alcohol and benzene were added. All solvents were evaporated to dryness under reduced pressure and the residue was chromatographed on 25 g of Celite using system G, holdback volume of 45 ml. The acetylated compound, eluted between seven and eleven holdback volumes, gave a positive methylene



FIGURE 4: ORD curves of 5-pregnene-3 β ,20 α -diol and 5-pregnene-3 β ,20 α -diol-20-N-acetylglucosaminide (III). $[m]_{\lambda}$ = molecular rotation at wavelength λ , $\Delta[m]_{\lambda}$ = molecular rotation difference.

blue test thus proving that the sulfate group had been retained. Since this sulfated product did not crystallize easily, it was converted into its pyridinium salt which, in turn, was solvolyzed in anhydrous tetrahydrofuran at room temperature for 24 hr (McKenna and Norymberski, 1957). After evaporation of the solvent, the solvolyzed product was chromatographed on 25 g of Celite using system H, holdback volume of 45 ml. The acetylated compound, eluted between 1.5 and 2.5 holdback volumes, crystallized from methanol and 1 or 2 drops of water, affording needles which melted at 252–254°.

Anal. Calcd for the triacetate of compound III, $C_{35}H_{33}NO_{10} \cdot 2H_2O$: C, 61.47; H, 8.40; N, 2.05. Found: C, 61.79; H, 8.02; N, 2.21.

This result was confirmed by acetylation of another sample of II with acetic-³H anhydride of high specific activity (sp act. 0.27 μ c/ μ mole). The triacetate was isolated in the manner described above. From the specific activity of the acetylated compound, recrystallized four times (467 cpm/ μ g), the number of acetylatable hydroxyl groups was found to be 3.15.

The ORD measurements were performed over the spectral region 546–230 m μ . Calibrated cylindrical

silica cells of 1.000 cm were used. All measurements were made at room temperature which was maintained between 22 and 24°. Figure 4 shows the ORD curves for 5-pregnene- 3α ,20 β -diol (c, 1.944 × 10⁻³ in methyl alcohol) and 5-pregnene- 3α ,20 β -diol-20-(2'-acetamido-2'-deoxy- α -D-glucoside) (c, 1.000 × 10⁻³ in methyl alcohol). The optical rotation of 5-pregnene- 3β ,20 α -diol was measured in methyl alcohol, [α]²⁵_D – 54.7° (c, 9.72 mg in 3 ml of methyl alcohol), [m]_D – 174.2°.

Discussion

Only once before has a steroid conjugated with N-acetylglucosamine been isolated from natural sources. In 1964, Layne *et al.* injected estrone benzoate, subcutaneously, into rabbits and isolated from the glucuronidase-treated urine an N-acetylglucosamide conjugate of 17α -estradiol. The release of the steroid by a β glucosidase preparation (emulsin) led these workers to suggest that the conjugate was a β -glucoside. In a later paper, Layne (1965) proved that the conjugate was estra-1,3,5(10)-trien- 17α -yl-2'-acetamido-2'-deoxy- β -D-glucopyranosiduronic acid. Subsequently, Jirku and Layne (1965) investigated the *in vitro* formation

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FIGURE 5: Curve representing $\Delta[m]_{\lambda}$ calculated from the ORD of 17-ketoandrostan- 3α -yl-2'-acetamido-2'-deoxy- β -D-glucoside and the ORD of 3α -hydroxyandrostan-17-one (androsterone).

of this conjugate by homogenates of rabbit liver.

The isolation of II reveals, for the first time, the presence of a glucosaminide conjugate of steroids in human urine. The results show that even under normal circumstances the quantity of such conjugates may not be entirely negligible. The isolation of "mixed conjugates" of steroids, i.e., metabolites conjugated with two different groups, has been reported before. The first of such compounds to be isolated was estriol-3-sulfate-16-glucuronide (Diczfalusy et al., 1964), while the second example was the 17α -estradiol-3glucuronide 17-N-acetylglucosaminide referred to above. Compound II is the only steroid conjugate ever characterized in which the glycosidic bond has been shown to be α . In fact, the stereochemistry of the glycosidic linkage of the many steroid glucuronides isolated from urine has received little attention. In a few instances, the assignment has been made by comparison with synthetic samples, the stereochemistry of which is well established. However, in most cases, this bond has been assumed to be β , because the conjugate was hydrolyzed by β -glucuronidase preparations. This conclusion appeared to be justified since Fishman and Bernfeld (1955) had shown that highly purified (1500fold) β -glucuronidase did not cleave α -glucuronides. However, it is undoubtedly true that crude β -glucuronidase preparations made from mammalian tissues contain many hydrolytic enzymes. For example, both β -Nacetylglucosaminidase and α -N-acetylglucosaminidase have been prepared from these sources (Roseman

and Dorfman, 1951; Weissmann *et al.*, 1964, 1967). In addition, Burstein and Dorfman (1962) have also shown that even the sulfates of 3β -hydroxy-5-ene steroids can be split under the usual conditions of incubation (37° at pH 4.5–5.5 for 3–5 days) in the absence of any enzyme preparation. Therefore, it would seem unwise to rely upon cleavage by crude glucuronidase preparations to provide proof of structure of steroid conjugates.

Optical rotation measurements provided the means for assigning the stereochemical configuration of the glycosidic bond between the hexosamine and the steroid. Foster and Stacey (1952) pointed out that the contribution of an α -N-acetylglucosamine residue to the molar rotation of a glycoside containing this residue was strongly positive while that of a β -linked hexosamine was negative. Thus, the molecular rotation of methyl-*N*-acetyl- α -D-glucosaminide is +246° while that of methyl-*N*-acetyl- β -D-glucosaminide is -101° . Since the $[m]_D$ for 5-pregnenediol is -175° , the contribution of the hexosamine moiety to III, whose $[m]_{d}$ is -95° , is $+80^\circ$. The assignment of an α configuration to the glycosidic bond was confirmed by ORD measurements. Beychok and Kabat (1965) have shown that β -linked N-acetylhexosaminides have a strong negative Cotton effect at about 225 m μ whereas α compounds do not. The absence of a Cotton effect in the ORD curve of III, as well as the fact that $\Delta[m]_{\lambda}$ becomes increasingly positive when approaching the lower wavelengths (Figure 4) gives strong support for the α configuration of the hexosaminidic bond in II and III.

Two reports have appeared which indicate that steroid disulfates, other than the one reported here, may be found in human urine. Following the administration of adrenocorticotrophin to normal subjects, Pasqualini and Jayle (1962) isolated from urine a material whose chromatographic and electrophoretic properties were identical with those of a synthetic sample of the disulfate of 3β ,21-dihydroxy-5-pregnen-20-one. Cleavage of the urinary conjugate yielded the steroid dihydroxy ketone which was identified by its infrared spectrum. In addition, following the administration of radioactive dehydroisoandrosterone sulfate to normal subjects, Baulieu and Corpechot (1965) recovered from urine a radioactive product whose chromatographic properties coincided with those of the disulfate of 5-androstene-33,178-diol. Proof of structure was obtained by a reverse isotope dilution technique using an authentic carrier.

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Added in Proof

Since this manuscript was submitted for publication, we learned that Dr. David K. Fukushima of the Institute for Steroid Research of the Montefiore Hospital, Bronx, N. Y., synthesized 17-ketoandrostan- 3α -yl-2'-acetamido-2'-deoxy- β -D-glucoside. He generously made this compound available to us and its ORD and that of androsterone were measured. The $\Delta[m]_{\lambda}$ calculated from these measurements was plotted as shown in Figure 5. By comparing this plot with that shown in Figure 4 it is evident that the contribution of the N-acetylglucosamine in this synthetic glucosaminide is negative and compatible with the β configuration of the glycosidic bond. These results add further support to the assignment of the α configuration for the natural conjugate of 5-pregnenediol isolated in this study.

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