Isolation of 17α-Estradiol 17-β-D-Glucopyranoside from Rabbit Urine, and Its Synthesis and Characterization*

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ABSTRACT: Incubation of 17α-estradiol 3-glucuronide with rabbit liver microsomes in the presence of uridine diphosphate glucose, and subsequent removal of the glucuronic acid from C-3 with β-glucuronidase, yielded a monoconjugate which corresponded chromatographically with a radioactive compound found in small amounts in the β-glucuronidase-treated urine of a rabbit injected with 17-β-estradiol-6,7-^{3}H. This material was isolated in crystalline form from the urine of rabbits treated with large doses of estrone benzoate, and was identified as 17α-estradiol 17-β-D-glucopyranoside (Va) by chromatographic and spectroscopic techniques. The structure of this compound was confirmed by chemical synthesis. This is the first demonstration of the occurrence of a steroid glucoside in animals.

Rabbit liver microsomes *in vitro* can effect the transfer of *N*-acetylglucosamine from its uridine nucleotide to the 17α-hydroxyl group of 17α-estradiol 3-glucuronide (Jirku and Layne, 1965; Collins *et al.*, 1968). In the course of further work on the enzymic mechanism of this transfer, UDP-glucose was substituted for UDP-*N*-acetylglucosamine in the incubation media. This resulted in the formation, in low yield, of a double conjugate. It was at first thought that the UDP-glucose might have contained UDP-*N*-acetylglucosamine as a contaminant but, after enzymatic removal of the glucuronic acid from C-3 of the steroid, the remaining monoconjugate was shown to be separable from 17α-estradiol 17-β-*N*-acetylglucosaminide by thin-layer chromatography. This indicated that the conjugate might be a glucoside, and a search was made for the compound in rabbit urine since only very small amounts could be made in the *in vitro* system. This paper describes the isolation and characterization of 17α-estradiol 17-β-D-glucopyranoside (Va) from rabbit urine.

Results

Urine from a rabbit, which had been injected with 17β-estradiol-6,7-^{3}H of high specific activity, was treated with β-glucuronidase and a chromatographic search revealed the presence in the conjugate fraction of a minor radioactive constituent with the same polarity as the suspected 17α-estradiol 17-β-D-glucopyranoside obtained *in vitro*. Treatment of this material with almond emulsin released a radioactive compound which crystallized to constant specific activity with 17α-estradiol. Approximately 4 mg of the conjugate was isolated in crystallized form from the urine of four rabbits which had received large doses of estrone benzoate. The infrared spectrum of this material was reconcilable with the proposal that the conjugating moiety was a hexose, and the ultraviolet spectrum showed the absorption at 279–285 mμ characteristic of ring A phenolic steroids. The major water-soluble product obtained by mild acid hydrolysis of the conjugate was chromatographically identical with glucose in systems which provided an adequate separation of this sugar from mannose and galactose.

The acetylated conjugate, IIIa, was also characterized by spectroscopic techniques, using a total of 2.4 mg of material, and finally by proving its identity to synthetic material. Thus, the nuclear magnetic resonance spectrum (Table I) showed a proton pattern in the aromatic region of the spectrum which was typical of ring A aromatic steroids (Bhacca and Williams, 1964). This, together with a distinct doublet at 226 Hz (C-17β proton) and a methyl signal at 42 Hz (C-18 methyl), suggested an estradiol moiety in which the C-17 oxygen substituent was in the α orientation, since, in substituted estradiol-17β-steroids the 17α-proton occurs as an indistinct triplet and the C-18 methyl signal, being more deshielded than in the 17α series, is usually found slightly further downfield than 42 Hz (Bhacca and Williams, 1964).

Resonance signals arising from the acetates and ring protons of the sugar moiety were consistent with a fully acetylated β-D-glucopyranoside structure (Hall, 1964). Thus, a doublet centered at 270 Hz with a spacing of 6.5 Hz was indicative of C-1′,2′ vicinal protons having a *trans* diaxial relationship. Methyl signals at 121 Hz (two overlapping peaks) and 120 Hz were in the range for equatorial acetate groups, while methyl signals at 125 and 136 Hz could be attributed to the sugar C-6 acetate and steroidal C-3 acetate, respectively (Bhacca and Williams, 1964). In the mass spectrum of the acetylated conjugate, the molecular ion was found at *m/e* 644 and signals at *m/e* 331 and 313 could be ascribed to the sugar and steroidal moieties, respectively. An abundant peak at *m/e* 254 could result from 1,2 elimination of the 17α-sugar substituent and loss of the steroidal 3-acetate as a ketene fragment, by analogy with the result reported for isomeric 17α-alkyl-17a-hydroxy-*D*-homoestrogens (Budzikiewicz *et al.*, 1964).

As unequivocal proof of structure, the compound in question was synthesized by a Koenigs–Knorr reaction (Hough

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TABLE I: The Nuclear Magnetic Resonance Spectra of Peracetylated 17α- and 17β-Estradiol 17-β-D-Glucopyranosides.

Assignment	Chemical Shift (Hz)	
	E_2-3-OAc-17α-$GlAc_4$ (IIIa)	E_2-3-OAc-17β-$GlAc_4$ (IIIb)
Steroid moiety		
H-1	436 d[a]	436
H-2	409 m	413 m
H-4	405 m	408 m
H-17	226 d (J = 5.5)	225 m
H-6	170 m	170 m
H-18	42 s	45 s
C-3-OAc	136 s	136 s
Sugar moiety		
H-2,3,4	304 m	306 m
H-1	270 d (J = 6.5)	276 d (J = 7.5)
H-6	249 m	253 m
H-5	220 m	220 m
C-6-OAc	125 s	125.5 s
C-2,3,4-OAc	121 s, 121 s, 120 s	123.5 s, 121.5 s, 121 s

[a] Refers to multiplicity of the chemical shift, *e.g.*, s = singlet, d = doublet, m = multiplet.

and Richardson, 1967) from 17α-estradiol 3-acetate (Ia) and acetobromoglucose (II) (Redemann and Niemann, 1955) using silver carbonate (Conchie and Levvy, 1963) as the condensing agent (Figure 1). The product, IIIa, was obtained in a yield of 25% together with an equal amount of the corresponding orthoacetate, IVa. Orthoacetates are frequently obtained as by-products in the Koenigs–Knorr reaction (Hall, 1964). In addition to its extreme acid lability, the orthoacetate IVa was readily identified by its nuclear magnetic resonance spectrum which showed a distinctive orthoacetyl C-methyl signal at 103 Hz (Mazurek and Perlin, 1965; Lemieux and Morgan, 1965). Mild acid hydrolysis of the reaction mixture, followed by chromatography on silica gel and crystallization from ethanol, gave the product IIIa as colorless crystals melting at 140–142°. The infrared, nuclear magnetic resonance, and mass spectra of this compound were identical in every respect with the natural peracetylated product except for evidence of silicone grease contamination in the latter. In addition, the polarity of both compounds were identical by thin-layer chromatography in two separate systems (20% acetone–hexane and 60% ether–hexane, silica gel plates, each developed three times).

Prior to synthesizing IIIa a trial reaction was carried out using the more readily available 17β-steroid, Ib (Fex *et al.*, 1968). The product, IIIb, was obtained in a yield of 57% together with a 16% yield of the corresponding orthoacetate IVb (Hall, 1964). Inasmuch as the polarity and spectral properties of the 17α and 17β products IIIa and IIIb are very similar, a comparison is given of their nuclear magnetic resonance spectra (Table I) as supportive evidence for the 17α configuration of the natural conjugate. In addition, the most abundant peak in the high mass region of the mass spectrum of IIIb occurs at *m/e* 255 (as compared with *m/e* 254 for the α isomer). Presumably, this is a reflection of the greater tendency of the β isomer to undergo cleavage of the 17β-oxygen function rather than being eliminated to give an unsaturated structure (Budzikiewicz *et al.*, 1964).

Compound IIIa was deacetylated in near quantitative yield by treatment with anhydrous ammonia in methanol (Conchie *et al.*, 1957) at 5° for 16 hr. Crystallization of the product, Va, from 33% aqueous ethanol gave microscopic colorless needles which melted indistinctly at 142–144° after drying in the Abderhalden. Elemental analysis of the hygroscopic product indicated the presence of 0.5 mole of water, and this was confirmed by Karl Fisher water analysis. Mass spectral analysis of the product showed a molecular ion peak ($[M]^+$ 434) which was surprisingly abundant for this type of molecule (Budzikiewicz *et al.*, 1964). The infrared and ultraviolet spectra of the product were identical with those of natural unacetylated conjugate.

Experimental Procedure

Experiments in Vitro. The preparation of rabbit liver microsomes and the incubation of these with 17α-estradiol 3-glucuronide in the presence of uridine nucleotides were carried out exactly as described by Collins *et al.* (1968). In a typical experiment 3 ml of the buffer described by these authors, containing 17α-estradiol-6,7-^{3}H 3-glucuronide at a concentration of 1.3×10^{-11} M and UDP-*N*-acetylglucosamine (Sigma) at a concentration of 6.7×10^{-6} M, was incubated for 60 min at pH 8.0. In a parallel tube UDP-glucose (Sigma) was substituted for UDP-*N*-acetylglucosamine. The yield of double conjugate in the two tubes was determined (Collins *et al.*, 1968) and was found to be 46% of the added glucuronide when UDP-*N*-acetylglucosamine was used, and 18% when UDP-glucose was substituted. The double conjugate from each tube was dissolved in 10 ml of 0.1 M sodium acetate buffer at pH 5.0, and 1 ml of Ketodase (Warner-Chilcott) was added. The tubes were incubated at 37° for 24 hr and the buffer was then extracted three times with ethyl acetate. The ethyl acetate extracts were chromatographed on thin-layer silica gel H (Merck) in chloroform–ethanol (4:1). The extract from the incubation with UDP-*N*-acetylglucosamine contained a monoconjugate with the same R_F (0.21) as 17α-estradiol 17-β-*N*-acetylglucosaminide (Layne *et al.*, 1964) while that from the incubation with UDP-glucose contained radioactive material with an R_F of 0.46.

Isolation of 17α-Estradiol 17-β-D-Glucopyranoside from Rabbit Urine. In a pilot experiment an extract of the urine from a female New Zealand rabbit which had received 10 mCi of 17β-estradiol-6,7-^{3}H (specific activity 5.6 Ci/mmole) was treated with Ketodase as previously described (Collins *et al.*, 1967) and the monoconjugate fraction was examined by thin-layer chromatography on silica gel H in chloroform–ethanol (4:1). In addition to 17α-estradiol 17-β-*N*-acetylglucosaminide, very small amounts of a radioactive material were found with the same R_F as the suspected 17α-estradiol 17-β-D-glucopyranoside formed *in vitro*. The ratio of this material to 17α-estradiol 17-β-*N*-acetylglucosaminide was approximately 1:100. If the urine was extracted prior to treatment with Ketodase, or if the Ketodase treatment was carried out in the presence of saccharo-1,4-lactone, the mono-

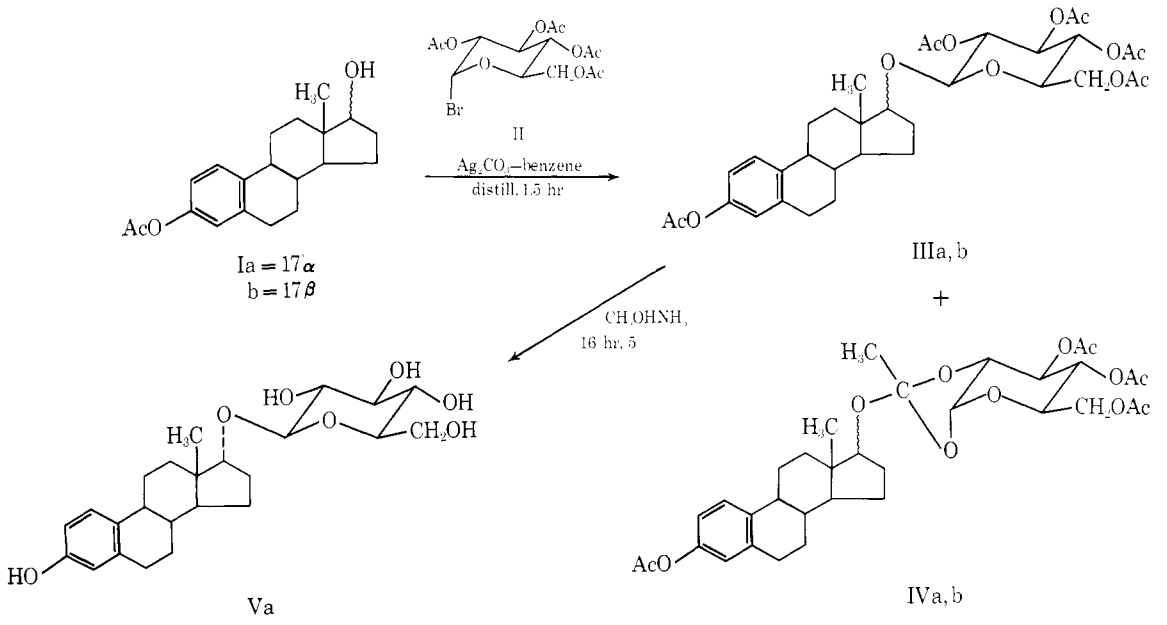

FIGURE 1: The synthesis of 17α-estradiol 17-β-D-glucopyranoside and related isomeric compounds.

conjugate was not detected. To test the stability of the monoconjugate to β-glucuronidase, 45,000 dpm of the material was incubated with 1 ml of Ketodase in 0.1 M sodium acetate buffer at 37° for 24 hr. Only 4% of the radioactivity was extractable from the buffer with benzene after the incubation.

Four female New Zealand rabbits were housed in metabolism cages and injected subcutaneously at multiple sites on each of 7 successive days with 100 mg of estrone benzoate in sesame oil. Urine was collected at 24-hr intervals for 12 days. The pooled urine was divided into 2-l. batches and each batch was applied to a column of Amberlite XAD-2 resin (Rohm and Haas, Toronto). The column was eluted with water and then with methanol exactly as described by Bradlow (1968). Each methanol extract was evaporated; the residue was dissolved in 250 ml of 0.1 M sodium acetate buffer at pH 5.0 and extracted three times with an equal volume of ethyl acetate. The ethyl acetate extracts contained only traces of monoconjugates as judged by thin-layer chromatography. The aqueous residues were each incubated for 48 hr after the addition of 20 ml of Ketodase, and were then extracted three times with an equal volume of benzene and three times with an equal volume of ethyl acetate. The pooled ethyl acetate extracts were evaporated to yield a semicrystalline mass consisting largely of 17α-estradiol 17-β-*N*-acetylglucosaminide. This compound was recrystallized three times from aqueous methanol. The combined mother liquors were examined on thin-layer silica gel H in chloroform–ethanol (4:1) and were found to contain the presumptive 17α-estradiol 17-β-D-glucopyranoside. After preparative thin-layer chromatography in the same system, this material was crystallized from aqueous methanol. The crystals melted at 139–143°.

Preliminary Characterization of Isolated Conjugate. A sample of 100 μg of the crystalline urinary conjugate was dissolved in 10 ml of 0.1 M sodium citrate at pH 4.3 containing 10 mg of almond emulsin (Sigma). After 24 hr at 37° the buffer was extracted with benzene and the benzene extract was examined on silica gel H in cyclohexane–ethyl acetate (7:3). Only a single spot was visible on spraying with 2% sulfuric acid in ethanol, and this had an R_F identical with that of 17α-estradiol. An aliquot of 89,100 dpm of the conjugate, obtained from the urine of the rabbit which received 17β-estradiol-6,7-^{3}H, was incubated with almond emulsin as described above. The benzene extract of the buffer after incubation contained 74% of the radioactivity. This material was mixed with 50.01 mg of reference 17α-estradiol and crystallized three times from aqueous methanol. The results (Table II) show that constant specific activity was obtained after the first crystallization.

The nonradioactive conjugate (250 μg) was suspended in 0.5 ml of water and 0.5 ml of concentrated hydrochloric acid in a stoppered tube. The tube was incubated at 37° for 16 hr, and the contents were then shaken with 2 ml of benzene. The benzene was removed by aspiration, 4 ml of ethanol was added to the aqueous phase, and the contents of the tube were evaporated under reduced pressure. The residue was dissolved in a few drops of ethanol–water (7:3). Half of the dissolved material was chromatographed on thin-layer silica gel H in 1-propanol–water (7:1) as described by Gal (1968). The plate was sprayed with an aqueous mixture of ammonium sulfate

TABLE II: Crystallization with 17α-Estradiol of the Aglycone of the Tritiated Conjugate from Rabbit Urine.[a]

	Sp Act. (dpm/mg)	
Crystzn	Crystals	Mother Liquor
1	1041	1577
2	1040	1299
3	1054	1198

[a] Calculated specific activity 1202 dpm/mg.

(20%) and sulfuric acid (4%). On heating for 20 min at 140° a single spot was observed at R_F 0.58. The R_F values of reference materials on parallel channels were: glucose, 0.58; galactose, 0.47; and mannose, 0.56. The remainder of the material obtained by acid hydrolysis of the conjugate was chromatographed on silica gel H in methyl acetate–isopropyl alcohol–water (18:1:1) and sprayed as described above. A single spot was observed at R_F 0.68. Corresponding values for the reference sugars were glucose 0.67, galactose 0.49, mannose 0.63.

Synthesis of 17α-Estradiol 17-β-D-Glucopyranoside and Related Isomeric Compounds. GENERAL. Organic solutions were dried over anhydrous sodium sulfate, filtrations were through a small bed of Celite (diatomaceous silica, Johns-Manville Co.), and evaporations were under reduced pressure on a rotary evaporator, unless otherwise indicated. Melting points were determined on a Mel-Temp apparatus in open capillaries and are corrected. Infrared spectra were determined in pressed potassium bromide disks on a Perkin-Elmer Model 21 spectrophotometer. Ultraviolet spectra were determined on a Cary Model 11 recording spectrophotometer. Optical rotations were measured on a Perkin-Elmer Model 141 polarimeter. Nuclear magnetic resonance spectra were determined on a Varian A-60 spectrometer with tetramethylsilane as internal standard. In determining the nuclear magnetic resonance spectra of IIIa on a micro scale (2.4 mg) a CAT computer (Hall, 1964) was used to increase the signal to noise ratio. Mass spectra were determined on an Associated Electrical Industries MS-9 instrument.

17α-Estradiol 3-monoacetate (Ia) was prepared from 17α-estradiol and aqueous sodium hydroxide–acetic anhydride as described by Fex *et al.* (1968) for the corresponding 17β compound. The product was obtained in a yield of 83% and the analytical sample had the following properties: mp 152–155° (Et_2O–hexane); $[\alpha]_D^{25}$ ($CHCl_3$) +47.6°; ν_{max}^{KBr} 3521 (OH), 1745, (CO), 1232 (COC), 1101 cm^{-1}; λ_{max}^{MeOH} 268 mμ and 276 mμ (ε 750); nuclear magnetic resonance ($CDCl_3$) 436 (d, H-1), 410 (m, H-2,4), 227 (d, H-17, J = 5.5 Hz), 170 (m, H-6,6), 135.5 (s, OAc), 93 (s, OH), 41 Hz (s, H-18); mass spectrum m/e 314 $(M)^+$, 272 (M − ketene).

Anal. Calcd for $C_{20}H_{26}O_3$ (314.41): C, 76.4; H, 8.34. Found: C, 76.42; H, 8.33.

3-Acetoxyestra-1,3,5(10)-trien-17α-yl 2′,3′,4′,6′-Tetra-O-acetyl-β-D-glucopyranoside (IIIa). A mixture of 943 mg (3.0 mmoles) of 17α-estradiol 3-monoacetate (Ia), 1.65 g (6.0 mmoles) of freshly prepared silver carbonate (Conchie and Levvy, 1963), and 60 ml of dry benzene was distilled until 15 ml of benzene had been removed. A solution of 2.47 g (6.0 mmoles) of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (II) (Redemann and Niemann, 1955) (mp 85–89°, $[\alpha]_D^{25}$ +191°; lit. mp 88–89°, $[\alpha]_D$ +197.8°) in 60 ml of dry benzene was added dropwise to this mixture over 1 hr with efficient stirring, during which 60 ml of benzene was simultaneously distilled from the flask. Half way through the addition of the bromo sugar a further 1.65 g of silver carbonate was introduced. When all of the bromo sugar had been added, the reaction was continued for 0.5 hr during which 30 ml of benzene was distilled and an equal volume of benzene was added. The mixture was filtered and evaporated to a yellow oil. Thin-layer chromatography showed that three main steroid-containing components were present. In an identical reaction, on a 1.0-mmole scale, these components were isolated by preparative thin-layer chromatography (three plates 20 × 20 cm with 2.0-mm layer of silica gel F-254, E. Merck AG, developed three times with 30% acetone–hexane followed by chromatography on eight plates, 20 × 20 cm with 0.5-mm layer of Mallinckrodt Silic AR TLC-7GF developed three times with 15% acetone–hexane) and were shown to consist of the desired product (IIIa), the corresponding orthoacetate[1] (IVa), and unreacted starting steroid (Ia) in a mole ratio of approximately 1:1:1.4, respectively.

The yellow oil was dissolved in dimethylformamide (27 ml), 0.1 N HCl (3.0 ml) was added, and the solution was allowed to stand at room temperature for 1.5 hr. The solution was poured into water (120 ml) and the mixture was filtered through Celite. The oily residue was washed on the filter with water, then dissolved in methylene chloride, and the resulting solution was evaporated. The product was co-evaporated several times with xylene to remove residual dimethylformamide, and the oily product (1.55 g) was chromatographed on silica gel (100 g of Mallinckrodt silic AR CC-7, 100–200 mesh). Elution of the column with 7.5% acetone–hexane provided starting steroid Ia (552 mg, 58%) which, on crystallization from methylene chloride–hexane, gave colorless crystals, mp 153–156°. Elution with 12% acetone–hexane gave 572 mg (29%) of product (IIIa) which, on crystallization from ethanol, furnished 487 mg (25%) of colorless crystals, mp 139–142°. The analytical sample was obtained by an additional crystallization from ethanol to give material with the following properties: mp 140–142°; $[\alpha]_D^{25}$ ($CHCl_3$) −15.0°; ν_{max}^{KBr} 1757 (CO), 1497 (aromatic), 1220 (COC), 1074, 1038 cm^{-1} (acetate); λ_{max}^{MeOH} 268 mμ and 276 mμ (ε 770); nuclear magnetic resonance ($CDCl_3$) 436 (d, H-1), 409 (m, H-2,4), 305 (m, H-2′,3′,4′), 270 (d, H-1′, J = 6.5 Hz), 250 (m, H-6′), 226 (d, H-17, J = 5.0 Hz), 220 (m, H-5′), 170 (m, H-6), 136 (s, C-3 OAc), 125 (s, C-6′ OAc), 120, 121 (s, C-2′,3′,4′ OAc), 42.5 Hz (s, H-18); mass spectrum m/e 644 $(M)^+$, 602 (M − ketene), 331 (sugar), 313 (steroid), 296 (M − sugar OH), 254 (M − sugar OH − ketene), 169 (sugar − 2HOAc − ketene).

3-Acetoxyestra-1,3,5(10)-trien-17β-yl 2′,3′,4′,6′-Tetra-O-acetyl-β-D-glucopyranoside (IIIb). The compound was prepared on a 1.0-mmole scale using the same procedure as for the 17α analog, IIIa. After filtration through Celite, the mixture was evaporated to an oil and purified by preparative thin-layer chromatography (two plates, 20 × 20 cm with 2.0-mm layer of silica gel F-254, E. Merck AG, developed

[1] Orthoacetate IVa [3-acetoxyestra-1,3,5(10)-trien-17α-yl-1′,2′-(methyl orthoacetyl)-3′,4′,6′-tri-*O*-acetyl-α-D-glucopyranose] was crystallized three times from ethanol and finally from ether–hexane to give colorless plates having the following properties: mp 159–169°; $[\alpha]_D^{25}$ ($CHCl_3$) +15.5°; ν_{max}^{KBr} 1754 (CO), 1497 (aromatic), 1222 (COC), 1040 (ester), 974 cm^{-1}; λ_{max}^{MeOH} 268 mμ and 276 mμ (ε 770); nuclear magnetic resonance ($CDCl_3$) 437 (d, H-1), 412 (m, H-2,4), 343.5 (d, H-1′, J = 5.0 Hz), 312 (m, H-3′), 296 (m, H-4′), 263 (m, H-2′), 253 (m, H-6′), 233 (m, H-17, H-5′), 170 (m, H-6), 136 (s, C-3 OAc), 127.5, 126 (s, C-3′,4′,6′ OAc), 103 (s, orthoacetate CH_3), 44 Hz (s, H-18); mass spectrum m/e 331 (sugar), 314 (steroid), 272 (steroid − ketene) 254 (M − sugar OH − ketene), 169 (sugar − 2HOAc − ketene). "Sugar" refers to the 1-deoxyglucopyranose moiety, "sugar O" refers to the 1-oxyglucopyranose moiety, and "sugar OH" refers to the 1-hydroxyglucopyranose moiety.

Anal. Calcd for $C_{34}H_{44}O_{12}$ (644.72): C, 63.34; H, 6.88. Found: C, 63.60; H, 6.89.

three times with 30% acetone–hexane). A wide band containing three ultraviolet-absorbing components was eluted and the product was crystallized twice from methylene chloride–ethanol. The product was filtered through a bed of Magnesol (4 g) using methylene chloride wash (20 ml) and crystallized again from methylene chloride–ethanol to give 268 mg of the desired product, IIIb, as colorless needles: mp 171–173°; $[\alpha]_D^{25}$ ($CHCl_3$) +17.3°; ν_{max}^{KBr} 1764 (CO), 1499 (aromatic), 1224 (COC), 1037 cm^{-1} (acetate); λ_{max}^{MeOH} 268 mμ and 276 mμ (ε 770); nuclear magnetic resonance ($CDCl_3$) 436 (d, H-1), 413 (m, H-2,4), 306 (m, H-2′,3′,4′), 276 (d, H-1′, J = 7.5 Hz), 253 (m, H-6′), 220 (m, H-17, H-5′), 170 (m, H-6), 136 (s, C-3 OAc), 125.5 (s, C-6′ OAc), 123.5, 121.5, 121 (s, C-2′,3′,4′ OAc), 45 Hz (s, H-18); mass spectrum *m/e* 644 $(M)^+$, 602 (M — ketene), 313 (steroid), 331 (sugar), 297 (M — sugar O), 255 (M — sugar O — ketene), 169 (sugar — 2OAc — ketene).

Anal. Calcd for $C_{34}H_{44}O_{12}$ (644.72): C, 63.34; H, 6.88. Found: C, 63.48; H, 6.82.

Mother liquors from crystallization of the product were evaporated and the residue purified by thin-layer chromatography (six plates 20 × 20 cm, 0.5-mm layer silica gel GF Analtech Inc., developed three times with 20% acetone–hexane). Three compounds were isolated from the plates: starting steroid Ib (45 mg, 14.6%, mp 130–141°), orthoacetate IVb[2] (118 mg, 15.6%, glass), and product IIIb (101 mg, glass). Thus the total yield of product was 369 mg (57.3%).

3-Hydroxyestra-1,3,5(10)-trien-17α-yl β-D-Glucopyranoside (Va). A solution of 355 mg (0.55 mmole) of estradiol 17α-glucopyranoside pentaacetate (IIIa) in 1 ml of methylene chloride and 15 ml of methanol was saturated with anhydrous ammonia at 5° (ice bath) and allowed to stand overnight (16 hr) in the refrigerator (+4°). The clear solution was evaporated to a colorless glass which was triturated with water to dissolve by-product acetamide. The solidified product was filtered and washed with water to give 235 mg (98%) of colorless solid. This material was crystallized twice from 33% aqueous ethanol to provide analytical material (223 mg) as microscopic colorless needles, mp 142–144° (crystals fuse), 149–153° (liquifies), after drying overnight at 80° over phosphorus pentoxide: $[\alpha]_D^{25}$ (MeOH) −2.7°; ν_{max}^{KBr} 3367 (OH), 1504 (aromatic), 1073, 1020 cm^{-1} (hydroxyl CO); λ_{max}^{MeOH} 282 mμ (ε 2000); nuclear magnetic resonance (d_6-acetone) 426 (d, H-1), 398 (m, H-2,4), 260 (d, H-1′, J = 7.0 Hz), 230, 203 (m, sugar ring protons, H-17), 183 (s, OH), 44 Hz (s, H-18); mass spectrum *m/e* 434 $(M)^+$, 255 (M — sugar O), 271 (steroid).

Anal. Calcd for $C_{24}H_{34}O_7 \cdot 0.5H_2O$ (443.54): C, 65.00; H, 7.96; H_2O, 2.03. Found: C, 64.86; H, 8.31; H_2O, 2.20.

[2] Orthoacetate IVb [3-acetoxyestra-1,3,5(10)-trien-17β-yl-1′,2′-(methyl orthoacetyl)-3′,4′,6′-tri-*O*-acetyl-α-D-glucopyranose] still contained minor impurities and could not be crystallized. It was purified again by thin-layer chromatography (four plates, 20 × 20 cm 0.5-mm layer silica gel GF, Analtech Inc., developed three times with 25% acetone–hexane) to give a glass which still resisted crystallization but was of analytical purity and had the following properties: $[\alpha]_D^{25}$ ($CHCl_3$) +51.2°; ν_{max}^{KBr} 1754 (CO), 1497 (aromatic), 1222 (COC), 1041 (acetate), 985 cm^{-1}; λ_{max}^{MeOH} 268 mμ and 276 mμ (ε 710); nuclear magnetic resonance ($CDCl_3$) 435 (d, H-1), 410 (m, H-2,4), 340 (d, H-1′, J = 5.5 Hz), 311 (m, H-3′), 295 (m, H-4′), 258 (m, H-2′, H-6′), 238 (m, H-5′), 218 (m, H-17), 170 (m, H-6), 135.5 (s, C-3 OAc), 127, 125 (s, C-3′,4′,6′ OAc), 103 (s, orthoacetate CH_3), 45.5 Hz (H-18).

Anal. Calcd for $C_{34}H_{44}O_{12}$ (644.72): C, 63.34; H, 6.88. Found: C, 63.02; H, 6.75.

Discussion

The results leave no doubt that the compound isolated from the urine of rabbits after the administration of estrogens was 17α-estradiol 17-β-D-glucopyranoside. The polarity and spectral properties of the fully acetylated derivative were essentially identical with material prepared synthetically, and the infrared and ultraviolet spectra of the deblocked compound matched those of the natural conjugate.

The 17α-estradiol 17-β-D-glucopyranoside was obtained from urine in significant amount only after treatment of the urinary extracts with β-glucuronidase (Ketodase). Further, the release of the monoconjugate by Ketodase was inhibited by the presence of saccharo-1,4-lactone. This indicates that, in the urine, the 3-hydroxyl of the steroid is also conjugated, probably with glucuronic acid. It is of interest that the glucoside is remarkably stable to β-glucuronidase, since it was cleaved to the extent of only 4% when incubated with Ketodase for 24 hr. By comparison, about 25% of a similar sample of 17α-estradiol 17-β-*N*-acetylglucosaminide is cleaved by Ketodase under these conditions (Layne *et al.*, 1964).

Glucoside formation from UDP-glucose is a well-known reaction in plants (see Williams, 1967). Sterols undergo this reaction (Eichenberger and Newman, 1968) and recently the 3 glucosidation of dehydroepiandrosterone by certain plant tissues has been demonstrated (Prochazka, 1968). In the animal kingdom insects (Smith and Turbert, 1964; Williams, 1967) and molluscs (Dutton, 1966) have been shown to transfer glucose from UDP-glucose to foreign compounds; the instances so far described have all involved transfer to a phenolic group. Basu *et al.* (1968) have detected a transferase in chick brain which transfers glucose from UDP-glucose to ceramide. Gessner and Vollmer (1969) obtained evidence for the glucosylation of *p*-nitrophenol by mouse liver microsomes but, with the exception of this observation, the 17α-estradiol 17-β-D-glucopyranoside isolated in the present work is, to the best of our knowledge, the first glucoside of a nonsugar found in mammals, and is the first such glucoside from an animal source to be fully characterized. The results indicate the presence in animal tissues of a novel glycosyl transfer mechanism, the characteristics and specificity of which remain to be explored. Whether this mechanism is related to those concerned in other metabolic reactions, such as glycogen synthesis, is of great interest, since the involvement of a steroid in such a reaction could be of considerable biochemical significance.

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Stereochemistry of Estrogen Biosynthesis*

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ABSTRACT: The stereoselective synthesis of [2β-^{3}H]- and [2α-^{3}H]androst-4-ene-3,17-diones is described. The orientation of the isotope in each followed from the method of preparation and from dehydrogenation with *Bacillus sphaericus* in which only the 2β-^{3}H was lost. Incubation of each substrate with human placental aromatase preparation established that the hydrogen loss from C-2 in estrogen biosynthesis is β. The recovered starting material showed a gain in tritium indicating the irreversibility of the loss and raising questions regarding the enolization mechanism proposed for this step. The 1β,2β stereochemistry of aromatization was further confirmed by the tritium analysis of estrone and 2-hydroxyestrone derived from [1,2-^{3}H]testosterone by sequential aromatization and 2 hydroxylation.

The *in vivo* transformation of cholesterol to the C-18 female sex hormone estradiol proceeds by way of discrete hormonal stages. The final sequence in this biotransformation which is the conversion of an androgen into estrogen involves the loss of the C-19 methyl group and of a hydrogen atom from both C-1 and C-2. Extensive study of this biosynthetic step has revealed that hydroxylation of the methyl group precedes its expulsion and that the hydrogen loss from C-1 is stereospecifically β (Talalay, 1965; Townsley and Brodie, 1968). The nature and stereochemistry of hydrogen loss from C-2 during the biological aromatization was the purpose of this study.[1]

The design of the experiment required the separate preparation of androst-4-ene-3,17-diones stereospecifically labeled with tritium in the 2α and 2β orientations which would serve as substrates for the enzymatic aromatization. At the outset their preparation was constrained by the requirement that the introduction of the isotope proceed by reactions of known high stereoselectivity. In addition since the desired α-methylene-labeled ketones would be subject to isotope loss or epimerization by an enolization reaction it was essential to limit the opportunity for this by delaying the formation of the C-3 ketone to the final step of the synthetic sequence. These requirements were fulfilled by the reaction sequences depicted in Scheme I. Reaction of 3β-tosyloxy-5α,6α-epoxyandrostan-17-one (**1**) with Li_2CO_3 in dimethylacetamide gave the olefin **2** which on reduction with $LiAlH_4$ led to androst-2-ene-5α,17β-diol (**3**). Epoxidation of **3** provided the 2α,3α-epoxide **4** which on opening with lithium aluminum tritiide gave the 2β-tritioandrostane-3α,5α,17β-triol (**5**). Selective acetylation of the two secondary hydroxyls led to the diacetate **6**; dehy-

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[1] While this manuscript was in preparation a report on the stereochemistry of hydrogen loss at C-2 during aromatization has appeared. Although different procedures were employed, the results obtained were in excellent agreement with those reported in this paper (Brodie *et al.*, 1969).