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Studies on Metabolism of 3-Deoxysteroids. VIII. Isolation and Characterization of Conjugated Metabolites of 3-Deoxyestrone[1,2]

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Isolation and characterization of the conjugated metabolites formed from 3-deoxyestrone have been carried out. The urine of rabbit with a large dosage of the steroid was collected and extracted with *n*-butanol. The extract was in turn chromatographed on Sephadex LH-20 to give 2-hydroxy-3-deoxyestrone glucuronide (Conjugate I). This procedure, however, failed to separate the remaining conjugates and therefore the glucuronides were transformed into the acetate-methyl esters. Upon column chromatography on alumina 17α-estradiol 17-glucuronide (Conjugate II), 6β-hydroxy-3-deoxyestrone glucuronide (Conjugate III) and 16,17-epiestriol 3,17 (or 3,16)-diglucuronide (Conjugate IV) were isolated as their derivatives and the structures were elucidated by degradative means (see Chart 1). The biochemical significance of the occurrence of these conjugates has been discussed.

The metabolic fate of 3-deoxyestrone (DOE), which is widely used as a lipid-shifting drug,[4] appeared to be of interest to us. In a previous paper of this series the identification of five phenolic and three neutral metabolites isolated from the rabbit urine after administration of DOE has been reported.[5] The recent studies revealed that the conjugate of the physiologically active substance is not necessarily the end-product and in certain case still possesses rather an enhanced activity.[6] A particular interest in these respects prompted us to explore the significance of conjugation with DOE metabolites. The present paper deals with the isolation and characterization of the conjugated metabolites of DOE administered to rabbit.

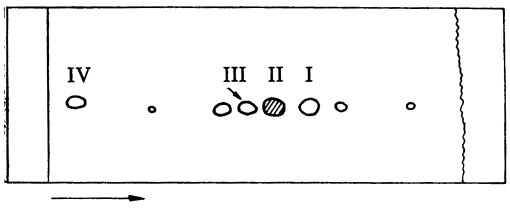

Fig. 1. Thin-Layer Chromatogram of the Conjugated Metabolites of 3-Deoxyestrone

adsorbent: Silica gel G (E. Merck AG)
developing solvent: $CHCl_3$–iso-PrOH–HCOOH (15:5:3)
staining reagent: conc. H_2SO_4
●: positive for Folin-Ciocalteu's test

A suspension of DOE in Tween 80 was orally given to an adult male rabbit, and the collected urine was extracted with ether and then with *n*-butanol. The butanol extract, whose thin-layer chromatography (TLC) was indicative of the existence of several conjugated methabolites (see Fig. 1), was digested with metanol. The precipitate hereby separated was then submitted to gel filtration on Sephadex LH-20 employing methanol as eluent. The eluate thus obtained was recrystallized to give Conjugate I as colorless plates.

1) This paper constitutes Part XLVI of the series entitled "Analytical Chemical Studies on Steroids," Part XLV: N. Shinriki and T. Nambara, *Yakugaku Zasshi*, in press.
2) The following trivial names are used: 3-deoxyestrone, estra-1,3,5(10)-trien-17-one; 17α-estradiol, estra-1,3,5(10)-triene-3,17α-diol; 16,17-epiestriol, estra-1,3,5(10)-triene-3,16β,17α-triol.
3) Location: *Aobayama, Sendai.*
4) A.H. Goldkamp, W.M. Hoehn, R.A. Mikulec, E.F. Nutting, and D.L. Cook, *J. Med. Chem.*, **8**, 409 (1965).
5) T. Nambara and M. Numazawa, *Chem. Pharm. Bull.* (Tokyo), **17**, 1200 (1969).
6) H.E. Hadd and R.T. Blickenstaff, "Conjugates of Steroid Hormones," Academic Press, 1969, p. 293; D.S. Layne, "Metabolic Conjugation and Metabolic Hydrolysis," ed. by W.H. Fishman, Academic Press, Vol. 1, 1970, p. 43.

Conjugate I gave a positive naphthoresorcinol test and underwent readily hydrolysis yielding aglycone and glucuronic acid when incubated with β-glucuronidase. The steroid moiety liberated was elucidated to be 2-hydroxy-3-deoxyestrone by TLC and mass spectrum exhibiting a fragment peak at *m/e* 269. Thus, Conjugate I was unequivocally identified as 2-hydroxy-3-deoxyestrone 2-glucuronide.

After removal of Conjugate I the remaining methanolic solution was chromatographed on Sephadex LH-20, but this procedure failed to separate each conjugate. Therefore the glucuronides were converted into the acetate–methyl ester derivatives by treatment with diazomethane and then with acetic anhydride and pyridine. These transformations were found to be effective for efficient separation by column chromatography on alumina.

The first eluate was purified by the preparative TLC and recrystallized from ether to give the acetate–methyl ester (II′) of Conjugate II as colorless needles. Inspection of the nuclear magnetic resonance (NMR) spectrum indicated the presence of three acetoxyl groups, methyl carboxylate and anomeric proton. The mass spectrum showed the fragments at *m/e* 313 and 317 assignable to aglycone and glucuronic acid moiety, respectively.[7] When treated with methanolic potassium hydroxide and then with beef–liver β-glucuronidase, II′ was led to a free steroid, which proved to be 17α-estradiol by comparison with the authentic sample on TLC. The methyl proton signal assignable to the phenol acetate of II′ appeared at 2.18 ppm and Conjugate II itself showed the positive reaction with Folin–Ciocalteu's reagent. Accordingly the attached position of glucuronyl group to the steroid nucleus was deduced to be C-17. These evidences together led to the conclusion that Conjugate II should be 17α-estradiol 17-monoglucuronide.

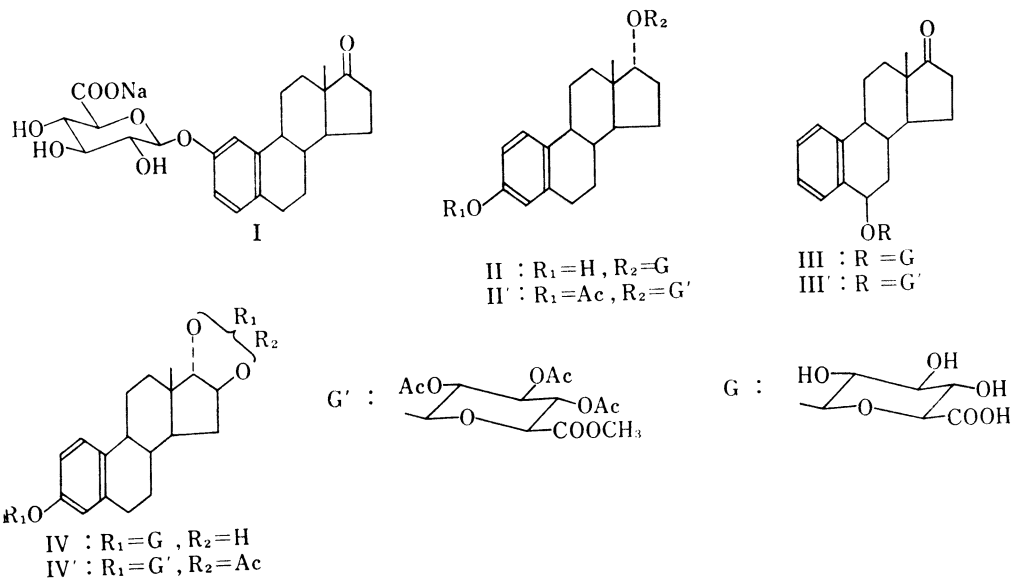

Chart 1

The acetate-methyl ester derivative (III′) of Conjugate III was then separated as colorless needles. The NMR spectrum indicated the presence of glucuronyl moiety exhibiting the signals at 1.99, 3.75 and 4.80—5.40 ppm. On the mass spectrum the characteristic fragment peaks, assignable to sugar aglycone and benztropylium ion, were observed at *m/e* 317, 270 and 141, respectively. Treatment with methanolic potassium hydroxide followed by enzy-

7) J. J. Schneider and N.S. Bhacca, *J. Org. Chem.*, **34**, 1990 (1970); T. Nambara, M. Numazawa, and Y. Matsuki to be published.

matic hydrolysis furnished 6β-hydroxy-3-deoxyestrone and glucuronic acid. Based upon these facts the structure of 6β-hydroxy-3-deoxyestrone 6-glucuronide was assigned to Conjugate III.

Finally, the acetate-methyl ester (IV') of Conjugate IV was afforded as colorless prisms. The NMR spectrum exhibited the methyl proton signals due to six O-acetates (2.04 ppm) and two methyl esters (3.71 and 3.73 ppm) suggesting the presence of two glucuronyl groups in the molecule. On the mass spectrum the fragment peaks characteristic to the acetate-methyl ester derivative appeared at *m/e* 645, 328 and 317. Upon treatment with methanolic potassium hydroxide Conjugate IV was recovered. Subsequent hydrolysis with β-glucuronidase liberated 16,17-epiestriol, which could be characterized by TLC. It was evident from the negative result for Folin–Ciocalteu's test that glucuronic acid should be attached to C-3 position. Although the definite evidence for the linked position in ring D of the remaining glucuronic acid moiety was unavailable, these data together permitted the assignment of the structure 16,17-epiestriol 3,17(or 3,16)-diglucuronide to Conjugate IV.

Discussion

In the preceding paper we reported that DOE was principally metabolized into 17α-estradiol, 2-hydroxy-3-deoxyestrone, 6β-hydroxy-3-deoxyestrone, 16,17-epiestriol and estrone in rabbits.[5,8] It is of interest that almost all the main metabolites are excreted as the conjugate with glucuronic acid.

As to the structures of the conjugated metabolites it is first to be noted that glucuronic acid is bound to the 6β-hydroxyl group through a glycoside linkage. To the best of our knowledge this appears to be the first reported conjugation at this position in the steroid nucleus. It has generally been accepted that an introduction of a hydroxyl group at C-6 in the estrogen molecule greatly enhances the polarity and the biochemical significance of the C-6 hydroxylic metabolite is related to this characteristic.[9] This explanation, however, appears to be inconsistent with the present finding that the C-6 hydroxylic steroid is still capable of conjugating with glucuronic acid, though DOE is an exogeneous substance.

The occurrence of Conjugate I, 2-hydroxy-3-deoxyestrone 2-glucuronide, is also noteworthy. Recently it was reported that metabolism of estrone by rat and hamster led to urinary excretion of 2-hydroxyestrone conjugated with glucuronic acid exclusively at C-2.[10] Independently, we also disclosed the excretion of the same conjugate in rat urine after administration of 2-hydroxyestorne.[11] The present result implies the existence of the enzyme system in rabbit which catalyzes the transfer of the glucuronyl residue to a phenolic hydroxyl function other than at C-3.

In addition, *in vivo* formafon of 17α-estradiol 17-monoglucuronide and 16,17-epiestriol 3,17(or 3,16)-diglucuronide should be emphasized. Layne and his co-workers reported the isolation of the double conjugates of 17α-estradiol, in which N-acetyl-D-glucosamine and D-glucose were attached to C-17 and D-glucuronic acid to C-3, from the urine of rabbit treated with a large dose of estrone benzoate.[12,13] They observed that the conjugation of N-acetyl-D-glucosamine with the 17α-hydroxyl group required the prior formation of 3-glucuronide or 3-sulfate of 17α-estradiol.[13,14] In connection with these findings it is of interest that the

8) T. Nambara and Y.H. Bae, *Chem. Pharm. Bull.* (Tokyo), **18**, 2119 (1970).
9) R. Knuppen, O. Haupt, and H. Breuer, *Biochem. J.*, **101**, 397 (1966); G.F. Marrian and A. Sneddon, *ibid.*, **74**, 430 (1960).
10) K.I.H. Williams, *Steroids*, **15**, 105 (1970).
11) T. Nambara and S. Honma, to be published.
12) D.G. Williamson, D.C. Collins, D.S. Layne, R.B. Conrow, and S.Bernstein, *Biochemistry*, **8**, 4299 (1969).
13) D.C. Collins, H. Jirku, and D.S. Layne, *J. Biol. Chem.*, **243**, 2928 (1968) and references quoted therein.
14) D.C. Collins and D.S. Layne, *Steroids*, **13**, 783 (1969).

glucuronic acid conjugation occurs at C-17 rather than at C-3 in 17α-estradiol. It has already been demonstrated by *in vitro* experiment that in rabbit liver the diglucuronide was formed with estriol and 17-epiestriol, but not with 16,17-epiestriol.[13] No plausible explanation for the difference between *in vivo* and *in vitro* results is now available. This may probably be due to the difference in breed.

Experimental

Animal——An adult male rabbit weighing about 2 kg was housed in a cage that was designed to minimize fecal contamination of urine.

β-Glucuronidase——Beef-liver preparation (Tokyo Zōkikagaku, Co.) was employed.

Administration of DOE and Collection of Urine——A single dose of suspension of DOE (700 mg/day) in Tween 80 was injected into a stomach through a catheter for 3 days and the urine was collected in a bottle containing a few drops of toluene for 5 days after the first administration.

Separation of Conjugated Metabolites——The pooled urine specimen (700 ml) was adjusted to pH 7 with dil.HCl and free steroids were removed by extraction with ether (500 ml×2). The aq.phase was saturated with NaCl (20 g/100 ml) and extracted with *n*-BuOH (800 ml×2). The *n*-BuOH phase was evaporated *in vacuo* below 50° to give a gummy residue (*ca.*4 g). The gummy substance was dissolved in MeOH (5 ml) and allowed to stand in refrigerator for 5 days. After separation of the precipitate (*ca.*200 mg) the filtrate was again allowed to stand for 7 days in refrigerator to yield the precipitate (*ca.*70 mg). These precipitates were combined and submitted to further purification (crude Conjugate I). The filtrate was evaporated to give a gummy residue (*ca.*3 g). This gummy substance was dissolved in MeOH (3 ml) and chromatographed on Sephadex LH-20 (2.5×50 cm) using MeOH as eluent. Elution was carried out at a rate of 5 ml/hr and fractions of 3 ml were collected. The effluent of fraction 8—11 was concentrated and then allowed to stand for 7 days in refrigerator. The precipitated Conjugate I (10 mg) was separated by filtration and the filtrate was rechromatographed on Sephadex LH-20 (2.5×50 cm). The effluent of fraction 8—14 was evaporated *in vacuo* to give a gummy residue (1.2 g). To a solution of this gummy substance in MeOH–CH_2Cl_2 (30 ml) was added an ethereal solution of CH_2N_2 and allowed to stand for 30 min at room temperature. After decomposition of the excess of CH_2N_2 with AcOH the resulting solution was evaporated to give an oily substance, which in turn was treated with Ac_2O (5 ml)–pyridine (10 ml) in the usual manner. The reaction mixture was diluted with ether and washed with 5% HCl, 5% $NaHCO_3$ and H_2O, successively and dried over anhydrous Na_2SO_4. After evaporation of solvent an oily residue (0.85 g) obtained was submitted to column chromatography on Al_2O_3 (20 g) using benzene–ether (1:4) (300 ml) as eluent. The first and last 50 ml portions of the effluent were discarded to remove the non-steroidal substances. The desired eluate was rechromatographed on Al_2O_3 (15 g) and fractions of 20 ml were collected as follows:

Fraction No.	Solvent system	Conjugate	Weight (mg)
1— 2	hexane–benzene (1:1)	II′	10
3— 9	benzene	II′, III′, IV′	85
10—15	benzene–ether (2:1)	III′, IV′	20

Separation of III′ and IV′ was achieved by preparative TLC on Silica gel H (E. Merck AG) using hexane–benzene (1:9) as solvent upon repeated developments.

Identification the Conjugated Metabolites[15]——**Sodium (17-Oxoestra-1,3-5(10)-trien-2-yl-β-D-glucopyranosid)uronate (Conjugate I)**: The precipitate (*ca.* 280 mg) described above was submitted to gel filtration on Sephadex LH-20 (2.5×50 cm) using MeOH as eluent and each 5 ml effluent was fractionally collected. The eluate of fraction 15—30 was rechromatographed on Sephadex LH-20 in the manner as mentioned above. Recrystallization of the eluate from aq. MeOH gave Conjugate I (45 mg) as colorless plates. mp 289.5—290.5° (decomp.). $[\alpha]_D^{20}$+50.2° (c=0.18, H_2O). *Anal.* Calcd. for $C_{24}H_{29}O_8Na \cdot 2\frac{1}{2}H_2O$: C, 56.13; H, 6.67; Na, 4.48. Found: C, 55.94; H, 6.63; Na, 4.65. TLC: *Rf* 0.60 ($CHCl_3$–iso-PrOH–HCOOH (15:5:3)). Naphthoresorcinol reaction[16] showed purple coloration (λ_{max} 562 mμ). A portion of Conjugate I (2.5 mg) was dissolved in acetate buffer (0.1 M, pH 4.8) and incubated with β-glucuronidase (13000 U) at 38° for 48

15) All melting points were taken on a micro hot-stage apparatus and are uncorrected. NMR spectra were recorded on Hitachi Model R-20 spectrometer using tetramethylsilane as an internal standard (TMS=0.00 ppm). Mass spectral measurements were run on Hitachi Model RMU-60 spectrometer. Infrared (IR) spectra were obtained by JASCO Model IR-S spectrophotometer.

16) W.H. Fishman and S. Green, *J. Biol. Chem.*, **215**, 527 (1955).

hr. The incubation mixture was extracted with ether and the extract was submitted to TLC using benzene–ether (1:1) as developing solvent. The hydrolyzate proved to be 2-hydroxy-3-deoxyestrone by comparison with the authentic sample (*Rf* 0.42) on TLC. IR ν_{max}^{KBr} cm^{-1}: 3300—3450 (OH), 1730 (5-membered ring C=O), 1620 (COO^-). Mass Spectrum *m/e*: 270, 269 (aglycone).

Methyl (3-Acetoxyestra-1,3,5(10)-trien-17α-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (II′) (Acetate-Methyl Ester of Conjugate II): Eluate was purified by preparative TLC using benzene as solvent upon repeated developments and recrystallized from ether to give II′ (10 mg) as colorless needles. mp 168—170°. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1730 (C=O). NMR ($CDCl_3$ solution) δ: 0.78 (3H, s, 18-CH_3), 1.98 (9H, s, pyranose-CH-OCOC**H**$_3$), 2.18 (3H, s, 3-OCOC**H**$_3$), 3.58 (3H, s, CH_3OCO-), 4.70—5.10 (3H, pyranose-C**H**-OAc), 4.75 (1H, d, J=6 cps, pyranose-C_1-H), 6.75—7.25 (3H, aromatic H). Mass Spectrum *m/e*: 317 (sugar moiety), 313 (aglycone). A portion of II′ (3 mg) was treated with 1N methanolic KOH at room temperature for 72 hr. After usual work-up Conjugate II recovered was dissolved in acetate buffer (0.1 M, pH 4.8) and incubated with β-glucuronidase (10000 U) at 38° for 48 hr. The incubation mixture was extracted with ether and the extract was submitted to TLC using benzene–ether (2:1) as developing solvent. The hydrolyzate proved to be 17α-estradiol by comparison with the authentic sample (*Rf* 0.38) on TLC.

Methyl (17-Oxoestra-1,3,5(10)-trien-6β-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (III′) (Acetate-Methyl Ester of Conjugate III): Eluate was purified by preparative TLC using benzene as solvent upon repeated developments and recrystallized from acetone to give III′ (5 mg) as colorless needles. mp 204—207°. NMR ($CDCl_3$ solution) δ: 0.84 (3H, s, 18—CH_3), 1.99 (9H, s, pyranose-CH-OCOC**H**$_3$), 3.75 (3H, s, CH_3OCO-), 4.80—5.40 (4H, pyranose-C**H**-OAc and -C_1-H), 7.20—7.40 (4H, aromatic H). Mass Spectrum *m/e*: 317 (sugar moiety), 270 (aglycone), 252 (aglycone–H_2O), 141 (benztropylium ion). A portion of III′ (1 mg) was treated with 1N methanolic KOH and then with β-glucuronidase (10000 U) in the manner as II′. The incubation mixture was extracted with ether and the extract was submitted to TLC using benzene–ether (2:1) as developing solvent. The hydrolyzate proved to be 6β-hydroxy-3-deoxyestrone by comparison with the authentic sample (*Rf* 0.35) on TLC.

Dimethyl [16β(or 17α)-Acetoxyestra-1,3,5(10)-trien-3,17α(or 16β)-ylene-di(2,3,4-tri-O-acetyl-β-D-glucopyranosid)]uronate (IV′) (Acetate-Methyl Ester of Conjugate IV): Eluate was purified by preparative TLC using benzene as solvent upon repeated developments and recrystallized from MeOH to give IV′ (15 mg) as colorless prisms. mp 217—220° (decomp.). NMR ($CDCl_3$ solution) δ: 0.76 (3H, s, 18-CH_3), 2.04 (21H, s, pyranose-CH-OCOC**H**$_3$ and 16(or 17)-OCOC**H**$_3$), 3.17 (3H, s, CH_3OCO-), 3.73 (3H, s, CH_3OCO-), 4.60—5.45 (8H, pyranose-C**H**-OAc and -C_1-H), 6.70—7.25 (3H, aromatic H). Mass Spectrum *m/e*: 645 (M^+–suger moiety), 328 (aglycone), 317 (suger moiety). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1735—1750 (C=O). A portion of IV′ (2 mg) was treated with 1N methanolic KOH in the manner as II′ to give Conjugate IV, which showed the negative result for Folin–Ciocalteu's reagent. Incubation with β-glucuronidase (10000 U) liberated 16, 17-epiestriol, which was identified by comparison with the authentic sample (*Rf* 0.40) on TLC using ether as developing solvent.

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