

Biliary Conjugated Metabolites of Estriol in the Rat¹⁾

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Isolation and characterization of the conjugated metabolites excreted in rat bile following the oral administration of a large dose of estriol has been undertaken. The principal conjugates (I—XI) were separated by chromatography on Amberlite XAD-2 resin, followed by gel filtration on Sephadex LH-20 and partition chromatography on silica gel. The structures of these metabolites were deduced from the physico-chemical data and definitely characterized by leading to the known derivatives and their comparison with the synthetic specimens. The physiological significance of biotransformation hereby observed has also been discussed.

Keywords—estriol; oral administration; fistula rat; biliary excretion; conjugated metabolite; isolation and characterization; catechol estrogen monosulfate; catechol estrogen monoglucuronide; glucuronide acetate-methyl ester; catechol O-methylation

Among the classic estrogens estriol is used as an orally administrative drug for the clinical states associated with the climacteric disorder. The metabolism of estrogen is characteristic of enterohepatic circulation, which is distinctly different from that of other steroid hormones.^{3,4)} The physiological significance of this metabolic feature, however, still remains unclear. In a previous paper of this series we reported the characterization of deconjugated biliary metabolites in the rat administered with estriol.⁵⁾ Further interest in these respects prompted us to explore the relationship between metabolic conjugation and biliary excretion. In this paper emphasis has been directed towards identification of the conjugated metabolites excreted in rat bile following the administration of a large dose of estriol.

A suspension of estriol in saline with Tween 80 was orally given to the fistula rat and bile was collected over a period of 48 hr after the administration. The pooled bile was diluted with water and percolated through a column of Amberlite XAD-2 resin. After thorough washing with distilled water the conjugate fraction was eluted with 60% methanol.⁶⁾ The eluate was then separated by partition chromatography on silica gel employing chloroform-methanol-water (70:30:6) as a mobile phase into six fractions (A-F). Further purification of each fraction by gel filtration on Sephadex LH-20 and partition chromatography on silica gel provided eleven conjugated metabolites (I—XI), which are numerically designated according to the order of increasing polarity (Chart 1).

The most nonpolar metabolite I, mp 252—255° (decomp.), was isolated as colorless needles. This substance resisted to hydrolysis with β -glucuronidase and showed a positive

- 1) Part CXX of "Studies on Steroids" by T. Nambara; Part CXIX: K. Shimada, Y. Fujii, E. Yamashita, Y. Niizaki, Y. Sato, and T. Nambara, *Chem. Pharm. Bull.* (Tokyo), **25**, 714 (1977). A preliminary account of this work has been presented: T. Nambara and Y. Kawarada, *Chem. Pharm. Bull.* (Tokyo), **23**, 698 (1975). In this paper the following trivial names were used: estriol, estra-1,3,5(10)-triene-3,16 α ,-17 β -triol; 16-epiestriol, estra-1,3,5(10)-triene-3,16 β ,17 β -triol; estradiol, estra-1,3,5(10)-triene-3,17 β -diol.
- 2) Location: *Aobayama, Sendai*.
- 3) P. Millburn, "Metabolic Conjugation and Metabolic Hydrolysis," Vol. II, ed. by W.H. Fishman, Academic Press, New York, 1970, pp. 45—50.
- 4) H. Watanabe, "Advances in Steroid Biochemistry and Pharmacology," Vol. 5, ed. by M.H. Briggs and G.A. Christie, Academic Press, New York, 1976, pp. 239—262.
- 5) T. Nambara, J. Ishiguro, Y. Kawarada, and H. Tajima, *Chem. Pharm. Bull.* (Tokyo), **22**, 889 (1974).
- 6) H.L. Bradlow, *Steroids*, **11**, 265 (1968).

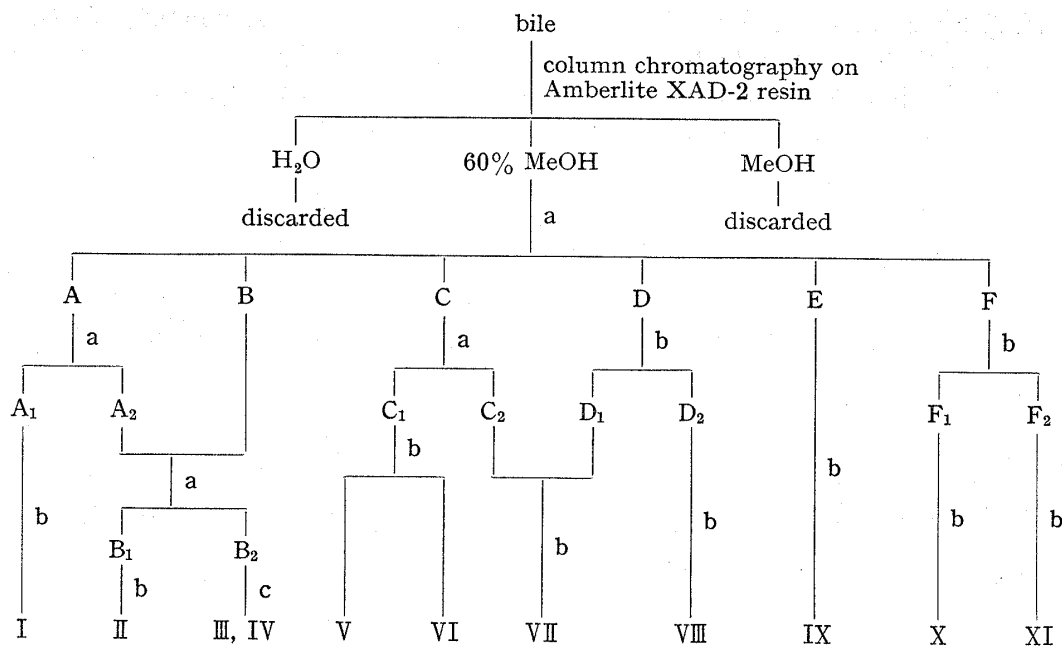


Chart 1. Separation of Conjugated Metabolites in Rat Bile following Administration of Estriol

a : partition chromatography on silica gel H solvent, CHCl_3 -MeOH- H_2O (70:30:6)
 b : gel filtration on Sephadex LH-20 solvent, H_2O
 c : mass chromatography

result with the Folin-Ciocalteu reagent. An infrared (IR) absorption at 1052 cm^{-1} due to the sulfonyl group and the positive barium-rhodizonate test⁷⁾ were indicative of metabolite I being a conjugate with sulfuric acid. The nuclear magnetic resonance (NMR) spectral data lent a support to assign the structure 2-hydroxy-16-epiestriol monosulfate. The conjugated position was determined by degradative means. Methylation with diazomethane, followed by hydrolysis with sulfuric acid furnished 2-hydroxy-16-epiestriol 3-methyl ether, which was identified by direct comparison with the authentic sample. On the basis of these evidences 2-hydroxy-16-epiestriol 2-sulfate was assigned to metabolite I.

Metabolite II, which was separated as colorless amorphous substance, showed the positive results for both the Folin-Ciocalteu and barium-rhodizonate tests. The NMR and IR spectral data indicated that the second metabolite would be 2-hydroxyestriol monosulfate. Elucidation of the conjugated position was similarly carried out and in consequence the formation of 2-hydroxyestriol 3-methyl ether was unequivocally demonstrated. It is evident from these data that 2-hydroxyestriol 2-sulfate should be assigned to metabolite II.

Recrystallization of the eluate of fr. B_2 gave a crystalline material. This substance showed the positive results with both the Folin-Ciocalteu reagent and naphthoresorcinol and liberated estriol when incubated with β -glucuronidase. As judged from the chromatographic behaviors the conjugate seemed to be homogeneous. However, careful inspection of the NMR spectrum of the acetate-methyl ester derivative revealed that this substance would be a mixture of estriol 16- and 17-glucuronides. The structures of these two metabolites (III, IV) were unambiguously characterized by means of mass chromatography with the O-trimethylsilyl ether-*n*-propyl ester derivatives.⁸⁾

Metabolite V was then separated as colorless amorphous substance. This compound exhibited the positive result for naphthoresorcinol and the negative Folin-Ciocalteu test.

7) D.P. Burma, *Anal. Chim. Acta*, **9**, 513 (1953); J.J. Schneider and M.L. Lewbart, *J. Biol. Chem.*, **222**, 787 (1956).

8) H. Miyazaki, M. Ishibashi, M. Itoh, N. Morishita, M. Sudo, and T. Nambara, *Biomed. Mass Spectrum.*, **3**, 55 (1976).

Hydrolytic cleavage with β -glucuronidase afforded 16-oxoestradiol supporting the assignment of 16-oxoestradiol 3-glucuronide to metabolite V. The complete structure was definitely established by leading to the known acetate-methyl ester derivative (1), mp 233–236°, by treatment with diazomethane and acetic anhydride-pyridine.

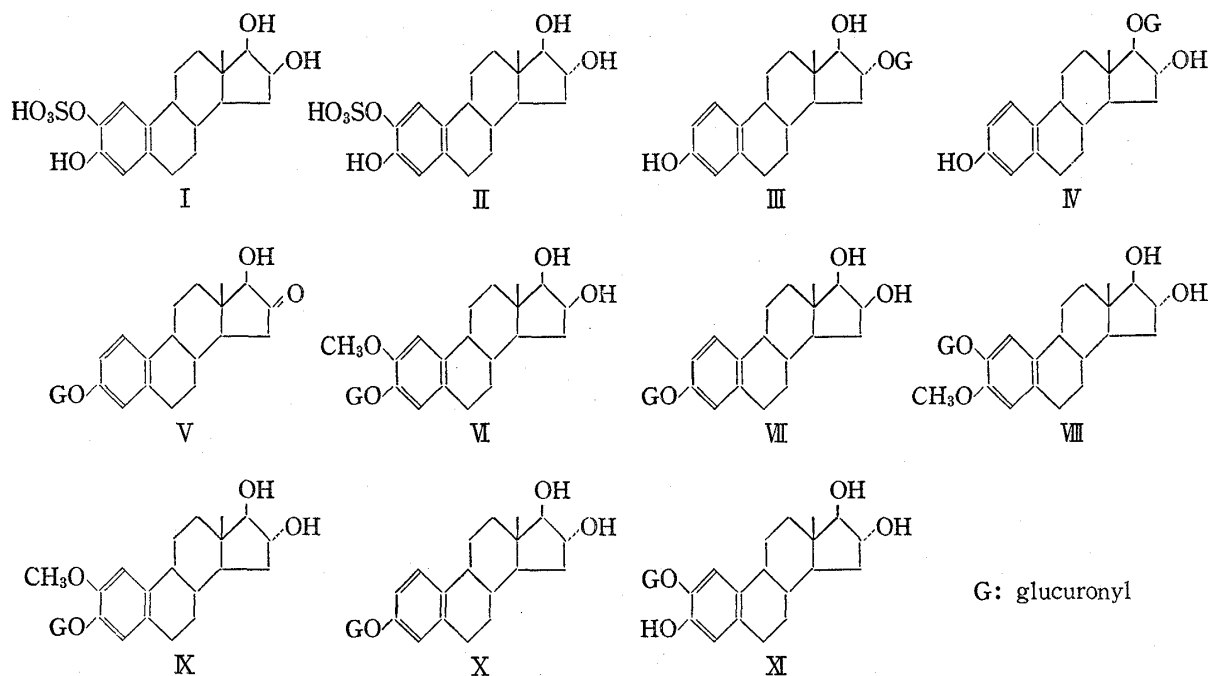


Chart 2. Conjugated Metabolites of Estriol in Rat Bile

Unfortunately metabolite VI could not be obtained in the crystalline state. According to the chromatographic behaviors, however, it was substantially homogeneous. The formation of 2-methoxy-16-epiestriol by enzymatic hydrolysis and the negative Folin-Ciocalteu test implied that metabolite VI would be 2-methoxy-16-epiestriol 3-glucuronide. In order to confirm the structure the metabolite was transformed into the acetate-methyl ester (2) in the usual manner. The NMR spectrum of this derivative exhibiting the signals due to an anomeric proton at 4.98 ppm ($J=7$ Hz) as a doublet and two aromatic ring protons at 6.80 ppm as a singlet rationalized the structural assignment.

Metabolite VII was separated from the eluate of fr. C₂ and D₁ by gel filtration on Sephadex LH-20. The color tests with naphthoresorcinol and the Folin-Ciocalteu reagent,

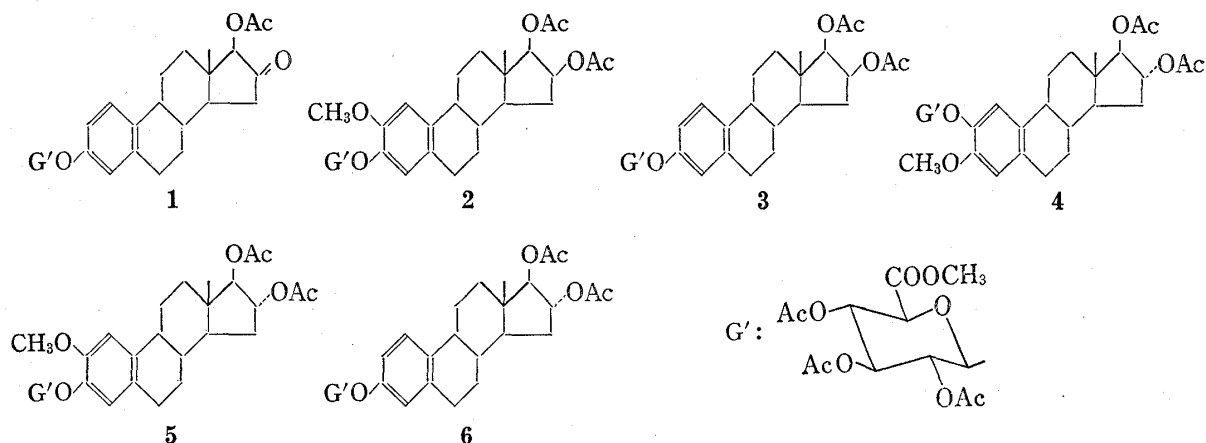


Chart 3. Glucuronide Acetate-Methyl Esters derived from Biliary Metabolites of Estriol

hydrolytic cleavage with β -glucuronidase, and transformation into the acetate-methyl ester derivative (3), mp 210—213°, justified to assign the structure 16-epiestriol 3-glucuronide to metabolite VII.

Metabolite VIII and IX having the similar chromatographic properties could be separated with success. Both the metabolites exhibited the positive test with naphthoresorcinol and the negative with the Folin-Ciocalteu reagent. The close similarity in the NMR spectra except the aromatic ring proton region strongly indicated that these two would be isomeric each other. In actuality hydrolysis with β -glucuronidase yielded 2-hydroxyestriol 3- and 2-monomethyl ethers. Derivatization of the metabolites into the acetate-methyl esters (4, 5), followed by direct comparison with the authentic specimens revealed that metabolite VIII and IX were 2-hydroxyestriol 3-methyl ether 2-glucuronide and 2-methoxyestriol 3-glucuronide, respectively.

Metabolite X showed the positive naphthoresorcinol test and underwent enzymatic hydrolysis to yield estriol. Treatment with diazomethane and subsequent acetylation provided the acetate-methyl ester (6), mp 194—197°, whose identity with the synthetic sample was verified by usual criteria. Thus the structure estriol 3-glucuronide was assignable to metabolite X.

The most polar metabolite XI exhibited the positive Folin-Ciocalteu and naphthoresorcinol tests. The NMR spectral data suggested the metabolite being 2-hydroxyestriol monoglucuronide. The attached position of the glucuronyl residue to the steroid nucleus was determined by leading to the known derivative. Methylation and subsequent acetylation in the usual manner afforded the acetate-methyl ester (4), mp 187—189°, which proved to be methyl (3-methoxy-16 α ,17 β -diacetoxyestra-1,3,5(10)-trien-2-yl-2,3,4-tri-O-acetyl- β -D-glucopyranosid)uronate by comparison with the authentic specimen. These evidences together permitted us to assign the structure 2-hydroxyestriol 2-glucuronide to metabolite XI.

In this study eleven kinds of conjugated metabolites excreted in rat bile following the oral administration of estriol have been unambiguously identified. The biliary metabolites were excreted principally in the form of glucuronic acid conjugate, although a small amount of the sulfate was also formed. The present finding is in accord with the result reported by Watanabe, *et al.*⁹⁾ It is to be noted that catechol estrogen was conjugated with the hydroxyl group predominantly in the aromatic ring rather than in ring D. These results strongly imply that C-2 hydroxylation, conjugation and possibly O-methylation with the phenolic group may be associated with facile excretion of the metabolites in bile. In humans uridine diphosphate-glucuronyltransferase is highly specific for the C-3 position of the catechol estrogen yielding solely the 3-glucuronide.¹⁰⁾ In sharp contrast metabolism of estrone by rats leads to excretion of urinary 2-hydroxyestrone conjugated with glucuronic acid at the C-2 position.¹¹⁾ The previous work in this laboratory demonstrated that 2-hydroxyestrone 2-glucuronide undergoes O-methylation at C-3 with retention of its glycoside linkage.¹²⁾ Hence it seems very likely that 2-hydroxyestriol 3-methyl ether 2-glucuronide may be formed principally from catechol estrogen 2-glucuronide. The *in vivo* formation of three possible estriol monoglucuronides is of interest indicating the multiplicity of uridine diphosphate-glucuronyltransferase in the rat.

This is the first demonstration of the occurrence of catechol estrogen monosulfate in the living animals. No evidence, however, was available for the biliary excretion of the isomeric 3-sulfate in the rat despite of much efforts. The present results together with the

9) H. Watanabe, P. Toft, and J.S. Menzies, *J. Steroid Biochem.*, **3**, 43 (1972); J.A. Menzies and H. Watanabe, *Steroids*, **27**, 595 (1976).

10) I. Yoshizawa and J. Fishman, *J. Clin. Endocrinol. Metab.*, **29**, 1123 (1969).

11) K.I.H. Williams, *Steroids*, **15**, 105 (1970).

12) S. Honma and T. Nambara, *Chem. Pharm. Bull.* (Tokyo), **23**, 787 (1975).

previous findings¹²⁻¹⁴) arrive at the assumption that catechol estrogen 3-sulfate may undergo O-methylation at the unoccupied phenolic group and subsequent transconjugation to yield 2-methoxyestrogen 3-glucuronide as a final product.

Clarification of some factors in the enterohepatic circulation of estrogens in respect with conjugation and deconjugation is a fertile field for further investigation.

Experimental

Animals—Male Wistar rats weighing 250 to 270 g were used. The rat was anesthetized with sodium pentobarbital, cannulated to the bile duct with polyethylene tube (PE 10, Clay Adams, Parsippany) by surgical operation, and housed in a Bollman cage for collection of bile.

Administration of Estriol—All animals were starved overnight prior to administration. A suspension of estriol (Teikoku Hormone Mfg. Co., Tokyo) (50 mg) in saline (0.75 ml) with Tween 80 (0.2 ml) was orally given to each of twenty rats and bile was collected over a period of 48 hr following administration.

Separation of Conjugated Metabolites—The bile samples were combined (350 ml), diluted with H₂O (2 liter), and percolated through a column (50 × 5 cm i.d.) packed with Amberlite XAD-2 resin (Rohm and Haas Co., Philadelphia). After thorough washing with distilled water (4 liter) the conjugate fraction was eluted with 60% MeOH. Evaporation of the solvent *in vacuo* below 40° gave a gummy residue, which in turn was submitted to partition chromatography on silica gel H (E. Merck AG, Darmstadt) (45 × 3.5 cm i.d.) using CHCl₃-MeOH-H₂O (70:30:6) as mobile phase. Elution was carried out at a rate of 10 ml/hr and each 10 ml fractionally collected was checked by TLC using CHCl₃-MeOH-H₂O (70:30:6) as developing solvent and conc. H₂SO₄ as spraying reagent. The conjugated metabolites were divided into six fractions from A to F with the increasing polarity. Further purification of each fraction by gel filtration on Sephadex LH-20 (Pharmacia Fine Chemicals AB, Uppsala) and partition chromatography on silica gel H gave eleven metabolites (I—XI) (Chart 1).

Identification of Metabolites¹⁵)

2-Hydroxy-16-epiestriol 2-Sulfate (Metabolite I)—The eluate of fr. A was submitted to gel filtration on Sephadex LH-20 (50 × 2.5 cm i.d.) using H₂O as solvent and each 5 ml was fractionally collected. The effluents of fr. 60—80 were combined and evaporated. Recrystallization of the eluate from H₂O gave metabolite I (5 mg) as colorless needles. mp 252—255° (decomp.). Folin-Ciocalteu test: positive; Ba²⁺-rhodizonate test: positive. NMR (D₂O solution) δ : 0.76 (3H, s, 18-CH₃), 3.39 (1H, d, $J=7$ Hz, 17 α -H), 4.13 (1H, m, 16 α -H), 6.63 (1H, s, 4-H), 7.15 (1H, s, 1-H). IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 1052 (SO₂). A portion of metabolite I (1.7 mg) was treated with CH₂N₂-ether (2 ml) overnight in a refrigerator. After addition of AcOH (1 drop) to decompose the excess CH₂N₂ the resulting solution was evaporated to dryness. The residue was dissolved in 5% H₂SO₄-MeOH (2 ml), allowed to stand at room temperature for 30 min, and then extracted with AcOEt. The organic layer was washed with 5% NaHCO₃ and H₂O, dried over anhydrous Na₂SO₄, and evaporated. Recrystallization of the residue from MeOH gave 2-hydroxy-16-epiestriol 3-methyl ether as colorless needles. mp 199—202°. Mixed melting point on admixture with the authentic sample¹⁶) showed no depression and IR spectra of two samples were entirely identical in every respect.

2-Hydroxyestriol 2-Sulfate (Metabolite II)—The eluate of fr. B₁ was further purified by gel filtration on Sephadex LH-20 in the manner as described in metabolite I. The effluents of fr. 40—50 were combined and evaporated to give metabolite II (10 mg) as colorless amorphous substance. Folin-Ciocalteu test: positive; Ba²⁺-rhodizonate test: positive. NMR (D₂O solution) δ : 0.71 (3H, s, 18-CH₃), 3.46 (1H, d, $J=6$ Hz, 17 α -H), 4.06 (1H, m, 16 β -H), 6.63 (1H, s, 4-H), 7.16 (1H, s, 1-H). IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 1042 (SO₂). A portion of metabolite II (2.1 mg) was treated with CH₂N₂-ether (2 ml) and then with 5% H₂SO₄-MeOH in the manner as described in metabolite I. Recrystallization of the crude product from MeOH gave 2-hydroxyestriol 3-methyl ether as colorless needles. mp 271—272°. Mixed melting point on admixture with the authentic sample¹⁷) showed no depression and IR spectra of two samples were entirely identical in every respect.

- 13) M. Miyazaki, I. Yoshizawa, and J. Fishman, *Biochemistry*, **8**, 1669 (1969); J. Fishman, I. Yoshizawa, and L. Hellman, *Steroids*, **22**, 401 (1973).
- 14) T. Nambara, S. Honma, and K. Kanayama, *Chem. Pharm. Bull.* (Tokyo), **20**, 2235 (1972).
- 15) All melting points were taken on a micro hot-stage apparatus and are uncorrected. IR spectra were obtained by a JASCO Model IR-S spectrometer. Mass spectral measurements were run on a Hitachi Model RMU-7 spectrometer. NMR spectra were recorded using tetramethylsilane and sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) (for the aqueous solution) as internal standards on Hitachi Model R-20A and JEOL Model PS-100 spectrometers at 60 MHz and 100 MHz, respectively. Abbreviation used s=singlet, d=doublet, and m=multiplet. Gas-chromatography-mass spectrometry was carried out on a Shimadzu Model LKB-9000s gas chromatograph-mass spectrometer equipped with a data-processing system.
- 16) T. Nambara and Y. Kawarada, *Chem. Pharm. Bull.* (Tokyo), **23**, 1613 (1975).
- 17) J. Fishman, M. Tomasz, and R. Lehman, *J. Org. Chem.*, **25**, 585 (1960).

Estriol 16-Glucuronide (Metabolite III), Estriol 17-Glucuronide (Metabolite IV)—The eluate of fr. B₂ was recrystallized from aq. MeOH to give a new metabolite (8 mg) as colorless needles. A portion of this substance was incubated with beef-liver β -glucuronidase (Tokyo Zōkikagaku Co., Tokyo) (1300 Fishman units) in 0.1M acetate buffer (pH 4.8) at 38° for 24 hr. The steroid aglycone liberated was extracted with AcOEt and identified as estriol by TLC. A portion of the metabolite (2 mg) was dissolved in MeOH (1 ml) and treated with CH₂N₂-ether solution (2 ml) for a brief period. After addition of AcOH to decompose the excess CH₂N₂ the resulting solution was evaporated. Treatment of the residue with Ac₂O-pyridine in the usual manner gave the acetate-methyl ester derivative. NMR (CDCl₃ solution) δ : 0.72, 0.77 (3H, s, 18-CH₃), 2.00–2.04 (12H, s, pyranose-OCOCH₃, 16 α -, 17 β -OCOCH₃), 2.26 (3H, s, 3-OCOCH₃), 3.74 (3H, s, pyranose-COOCH₃), 4.52 (1H, d, $J=8$ Hz, pyranose-1-H). Mass Spectrum m/e : 688 (M⁺), 646, 371, 369, 355, 317, 257, 253. The metabolite seemed to be homogeneous as judged from the chromatographic behaviors, but the NMR spectral data indicated that it would be a mixture of two isomeric glucuronides. The metabolite was converted to the O-trimethylsilyl ether-*n*-propyl ester derivative and submitted to mass chromatography under the conditions previously established.⁸⁾ The metabolite proved to be a mixture of estriol 16- and 17-glucuronides (III, IV) by comparison with the authentic sample.¹⁸⁾

16-Oxoestradiol 3-Glucuronide (Metabolite V)—The eluate of fr. C₁ was separated by gel filtration on Sephadex LH-20 in the manner as described in metabolite I to give metabolite V (4 mg) as colorless amorphous substance. Folin-Ciocalteu test: negative; naphthoresorcinol test: positive. NMR (CD₃OD solution) δ : 0.77 (3H, s, 18-CH₃), 3.80 (1H, s, 17 α -H). Incubation with β -glucuronidase gave 16-oxoestradiol, which was characterized by TLC. A portion of metabolite V (2 mg) was treated with CH₂N₂-ether (2 ml) at room temperature for 30 min. After addition of AcOH to decompose the excess CH₂N₂ the resulting solution was evaporated. The residue was then treated with Ac₂O-pyridine in the usual manner. Recrystallization of the crude product from MeOH gave methyl (16-oxo-17 β -acetoxyestra-1,3,5(10)-trien-3-yl-2,3,4-tri-O-acetyl- β -D-glucopyranosid)uronate (1) as colorless needles. mp 233–236°. NMR (CDCl₃ solution) δ : 0.86 (3H, s, 18-CH₃), 2.04 (9H, s, pyranose-OCOCH₃), 2.18 (3H, s, 17 β -OCOCH₃), 3.72 (3H, s, pyranose-COOCH₃), 4.12 (1H, m, pyranose-5-H), 5.08 (1H, s, 17 α -H), 5.00–5.40 (4H, m, pyranose-CH-OAc), 6.70 (1H, s, 4-H), 6.75 (1H, d, $J=8$ Hz, 2-H), 7.16 (1H, d, $J=8$ Hz, 1-H). Mass Spectrum m/e : 328, 317, 214, 213.

2-Methoxy-16-epiestriol 3-Glucuronide (Metabolite VI)—The eluate of fr. C₁ was further separated by gel filtration on Sephadex LH-20 to give metabolite VI (3 mg) as oily substance. Folin-Ciocalteu test: negative; naphthoresorcinol test: positive. Incubation with β -glucuronidase gave 2-methoxy-16-epiestriol,¹⁶⁾ which was characterized by TLC. Treatment with CH₂N₂-ether and then with Ac₂O-pyridine in the usual manner gave methyl (2-methoxy-16 β ,17 β -diacetoxyestra-1,3,5(10)-trien-3-yl-2,3,4-tri-O-acetyl- β -D-glucopyranosid)uronate (2) as oily substance. NMR (CDCl₃ solution) δ : 0.92 (3H, s, 18-CH₃), 2.03–2.06 (15H, s, 16 β -, 17 β -OCOCH₃, pyranose-OCOCH₃), 3.72 (3H, s, pyranose-COOCH₃), 3.76 (3H, s, 2-OCH₃), 4.07 (1H, m, pyranose-5-H), 4.61 (1H, d, $J=7$ Hz, 17 α -H), 4.98 (1H, d, $J=7$ Hz, pyranose-1-H), 5.14–5.40 (4H, m, 16 α -H, pyranose-CH-OAc), 6.80 (2H, s, 1-, 4-H). Mass Spectrum m/e : 718 (M⁺), 658, 402, 317, 258. NMR and IR spectra were entirely identical with those of the authentic sample.¹⁹⁾

16-Epiestriol 3-Glucuronide (Metabolite VII)—The eluate of fr. C₂ and D₁ was further purified by gel filtration on Sephadex LH-20 to give metabolite VII (10 mg) as colorless amorphous substance. NMR (D₂O solution) δ : 0.78 (3H, s, 18-CH₃), 3.44 (1H, d, $J=7$ Hz, 17 α -H), 3.51–3.94 (4H, m, pyranose-CH-OH, pyranose-5-H), 4.17 (1H, m, 16 α -H), 5.02 (1H, d, $J=7$ Hz, pyranose-1-H), 6.84 (1H, s, 4-H), 6.89 (1H, d, $J=8$ Hz, 2-H), 7.28 (1H, d, $J=8$ Hz, 1-H). Folin-Ciocalteu test: negative; naphthoresorcinol test: positive. Incubation with β -glucuronidase gave 16-epiestriol, which was characterized by TLC. Metabolite VII was treated with CH₂N₂-ether and then with Ac₂O-pyridine in the usual manner. Recrystallization of the crude product from MeOH gave methyl (16 β ,17 β -diacetoxyestra-1,3,5(10)-trien-3-yl-2,3,4-tri-O-acetyl- β -D-glucopyranosid)uronate (3) as colorless needles. mp 210–213°. NMR (CDCl₃ solution) δ : 0.93 (3H, s, 18-CH₃), 2.05 (15H, s, 16 β -, 17 β -OCOCH₃, pyranose-OCOCH₃), 3.73 (3H, s, pyranose-COOCH₃), 4.15 (1H, m, pyranose-5-H), 4.63 (1H, $J=7$ Hz, 17 α -H), 5.00–5.40 (5H, m, 16 α -H, pyranose-CH-OAc, pyranose-1-H), 6.71 (1H, s, 4-H), 6.75 (1H, d, $J=8$ Hz, 2-H), 7.17 (1H, d, $J=8$ Hz, 1-H). Mass Spectrum m/e : 688 (M⁺), 372, 317, 257. Mixed melting point on admixture with the authentic sample²⁰⁾ showed no depression, and IR and NMR spectra of two samples were entirely identical.

2-Hydroxyestriol 3-Methyl Ether 2-Glucuronide (Metabolite VIII)—The eluate of fr. D₂ was further purified by gel filtration on Sephadex LH-20 to give metabolite VIII (10 mg) as oily substance. Folin-Ciocalteu test: negative; naphthoresorcinol test: positive. NMR (D₂O solution) δ : 0.71 (3H, s, 18-CH₃), 3.59 (1H, d, $J=6$ Hz, 17 α -H), 3.76 (3H, m, pyranose-CH-OH), 3.91 (3H, s, 3-OCH₃), 4.17 (1H, m, pyranose-5-H), 5.03 (1H, d, $J=7$ Hz, pyranose-1-H), 6.74 (1H, s, 4-H), 7.10 (1H, s, 1-H). Incubation with β -glucuronidase gave 2-hydroxyestriol 3-methyl ether,¹⁷⁾ which was characterized by TLC. Metabolite VIII was treated with CH₂N₂-ether and then with Ac₂O-pyridine in the usual manner. Recrystallization

18) T. Nambara, Y. Kawarada, K. Shibata, and T. Abe, *Chem. Pharm. Bull.* (Tokyo), **20**, 1988 (1972).

19) T. Nambara and Y. Kawarada, *Chem. Pharm. Bull.* (Tokyo), **24**, 421 (1976).

20) T. Nambara, Y. Matsuki, and Y. Kawarada, *Chem. Pharm. Bull.* (Tokyo), **19**, 844 (1971).

of the crude product from acetone-hexane gave methyl (3-methoxy-16 α ,17 β -diacetoxyestra-1,3,5(10)-trien-2-yl-2,3,4-tri-O-acetyl- β -D-glucopyranosid)uronate (4) as colorless needles. mp 189—191°. NMR (CDCl₃ solution) δ : 0.85 (3H, s, 18-CH₃), 2.05—2.09 (15H, s, 16 α -, 17 β -OCOCH₃, pyranose-OCOCH₃), 3.72 (3H, s, pyranose-COOCH₃), 3.74 (3H, s, 3-OCH₃), 4.04 (1H, m, pyranose-5-H), 4.96 (2H, d, J =7 Hz, 17 α -H, pyranose-1-H), 5.00—5.40 (4H, m, 16 β -H, pyranose-CH-OAc), 6.57 (1H, s, 4-H), 7.06 (1H, s, 1-H). Mass Spectrum m/e : 718 (M⁺), 658, 402, 317, 257, 215. Mixed melting point on admixture with the authentic sample¹⁹ showed no depression, and IR and NMR spectra of two samples were entirely identical.

2-Methoxyestriol 3-Glucuronide (Metabolite IX)—The eluate of fr. E was purified by gel filtration on Sephadex LH-20 to give metabolite IX (20 mg) as colorless amorphous substance. Folin-Ciocalteu test: negative; naphthoresorcinol test: positive. NMR (D₂O solution) δ : 0.71 (3H, s, 18-CH₃), 3.59 (1H, d, J =6 Hz, 17 α -H), 3.75 (3H, m, pyranose-CH-OH), 3.90 (3H, s, 2-OCH₃), 4.18 (1H, m, 16 β -H), 5.10 (1H, d, J =7 Hz, pyranose-1-H), 6.92 (2H, s, 1-, 4-H). Incubation with β -glucuronidase gave 2-methoxyestriol,¹⁷ which was characterized by TLC. Treatment of metabolite IX with CH₂N₂-ether and then with Ac₂O-pyridine in the usual manner gave methyl (2-methoxy-16 α ,17 β -diacetoxyestra-1,3,5(10)-trien-3-yl-2,3,4-tri-O-acetyl- β -D-glucopyranosid)uronate (5) as oily substance. NMR (CDCl₃ solution) δ : 0.84 (3H, s, 18-CH₃), 2.03—2.10 (15H, s, 16 α -, 17 β -OCOCH₃, pyranose-OCOCH₃), 3.72 (1H, s, pyranose-COOCH₃), 3.75 (3H, s, 2-OCH₃), 4.08 (1H, m, pyranose-5-H), 4.97 (2H, d, J =6 Hz, 17 α -H, pyranose-1-H), 5.06—5.40 (4H, m, 16 β -H, pyranose-CH-OAc), 6.77 (1H, s, 4-H), 6.80 (1H, s, 1-H). Mass Spectrum m/e : 718 (M⁺), 658, 444, 402, 317, 257. IR and NMR spectra of 5 were entirely identical with those of the authentic sample.¹⁹

Estriol 3-Glucuronide (Metabolite X)—The eluate of fr. F₁ was further purified by gel filtration on Sephadex LH-20 to give metabolite X (5 mg) as colorless amorphous substance. Folin-Ciocalteu test: negative; naphthoresorcinol test: positive. NMR (D₂O solution) δ : 0.71 (3H, s, 18-CH₃), 3.52 (1H, d, J =6 Hz, 17 α -H), 3.53—3.97 (4H, m, pyranose-CH-OH, pyranose-5-H), 4.11 (1H, m, 16 β -H), 5.03 (1H, d, J =7 Hz, pyranose-1-H), 6.85 (1H, s, 4-H), 6.89 (1H, d, J =8 Hz, 2-H), 7.24 (1H, d, J =8 Hz, 1-H). Incubation with β -glucuronidase gave estriol, which was characterized by TLC. Metabolite X was treated with CH₂N₂-ether and then with Ac₂O-pyridine in the usual manner. Recrystallization of the crude product from MeOH gave methyl (16 α ,17 β -diacetoxyestra-1,3,5(10)-trien-3-yl-2,3,4-tri-O-acetyl- β -D-glucopyranosid)uronate (6) as colorless needles. mp 194—197°. NMR (CDCl₃ solution) δ : 0.84 (3H, s, 18-CH₃), 2.03—2.07 (15H, s, 16 α -, 17 β -OCOCH₃, pyranose-OCOCH₃), 3.69 (3H, s, pyranose-COOCH₃), 4.10 (1H, m, pyranose-5-H), 4.95 (1H, d, J =6 Hz, 17 α -H), 4.80—5.40 (5H, m, 16 β -H, pyranose-CH-OAc, pyranose-1-H), 6.68 (1H, s, 4-H), 6.72 (1H, d, J =8 Hz, 2-H), 7.14 (1H, d, J =8 Hz, 1-H). Mass Spectrum m/e : 688 (M⁺), 628, 372, 317, 257. Mixed melting point on admixture with the authentic sample²¹ showed no depression, and IR and NMR spectra of two samples were entirely identical.

2-Hydroxyestriol 2-Glucuronide (Metabolite XI)—The eluate of fr. F₂ was further purified by gel filtration on Sephadex LH-20 to give metabolite XI (5 mg) as colorless amorphous substance. Folin-Ciocalteu test: positive; naphthoresorcinol test: positive. NMR (D₂O solution) δ : 0.72 (3H, s, 18-CH₃), 3.49 (1H, d, J =6 Hz, 17 α -H), 3.50—3.96 (4H, m, pyranose-CH-OH, pyranose-5-H), 4.10 (1H, m, 16 β -H), 4.92 (1H, d, J =7 Hz, pyranose-1-H), 6.66 (1H, s, 4-H), 7.05 (1H, s, 1-H). Metabolite XI was treated with CH₂N₂-ether and then with Ac₂O-pyridine in the usual manner. Recrystallization of the crude product from acetone-hexane gave methyl (3-methoxy-16 α ,17 β -diacetoxyestra-1,3,5(10)-trien-2-yl-2,3,4-tri-O-acetyl- β -D-glucopyranosid)uronate (4) as colorless needles. mp 187—189°. Mixed melting point on admixture with the authentic sample¹⁹ showed no depression, and IR and NMR spectra of two samples were entirely identical.

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21) T. Nambara and K. Imai, *Chem. Pharm. Bull.* (Tokyo), **15**, 1232 (1967).