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Isolation and Characterization of Biliary Metabolites of Estriol in the Rat[1)]

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16-Oxoestradiol (IV), two pairs of isomeric 2- and 3-methyl ethers of 2-hydroxy-estriol (II, III) and 2-hydroxy-16-oxoestradiol (V, VI) have been characterized as biliary glucuronide metabolites of estriol in the rat. This is the first report on *in vivo* formation of catechol estrogen having the 16-oxo-17β-ol structure.

Of the classic estrogens, only estriol is widely used as an orally administrative drug for the clinical states associated with the climacteric disorders, senile vaginitis, and cervicitis. In recent years considerable attentions have been focused to the physiological significance of this female hormone in the feto-placental unit.[3)] The metabolism of estrogen is characteristic of enterohepatic circulation, which is distinctly different from other steroid hormones.[4)] A particular interest in these respects prompted us to clarify the estrogen metabolites excreted in bile. In a previous work of this series, we reported biliary excretion of the metabolites formed from estrone.[5)] The present paper deals with isolation and characterization of the biliary metabolites in the rat administered with estriol.

A single dose of a suspension of estriol in saline was orally given to the female rats with cannulation of the bile duct and bile was collected for following 6 hr. Separation of the glucuronide metabolites was processed in the manner as shown in Chart 1. Pooled bile was extracted with acetone–ethanol and the resulting precipitate was removed by centrifugation.[6)] The remaining phase was submitted to hydrolysis with β-glucuronidase to give the steroidal aglycones. Inspection by thin–layer chromatography (TLC) with system A indicated that the hydrolyzate would consist of four main fractions. However, fraction II and IV could be further separated into two metabolites, respectively. Both fraction I and III were substantially homogeneous providing solely the corresponding metabolites. These six metabolites derived from the glucuronide fraction are numerically designated according to the order of decreasing polarity.

Recrystallization of the most polar substance afforded metabolite I as colorless needles. In the mass spectrum a molecular ion peak appeared at m/e 288, accompanied with the fragment ion peaks due to ring A indicating the estratriene-3,16,17-triol structure. It seemed

1) This paper constitutes Part LXX of the series entitled "Analytical Chemical Studies on Steroids"; Part LXIX: M. Numazawa, Y. Tanaka, Y. Momono (née Hotta), and T. Nambara, *Chem. Pharm. Bull.* (Tokyo), 22, 663 (1974). In this paper the following trivial names were used: estrone, 3-hydroxy-estra-1,3,5(10)-trien-17-one; estradiol, estra-1,3,5(10)-triene-3,17β-diol; estriol, estra-1,3,5(10)-triene-3,16α,17β-triol.
2) Location: *a) Aobayama, Sendai*; *b) Kamiya, Kita-ku, Tokyo.*
3) E.-E. Baulieu, C. Corpechot, F. Dray, R. Emiliozzi, M.-C. Lebeau, P. Mauvais-Jarvis, and P. Robel, "Recent Progress in Hormone Research," Vol. 21, ed. by G. Pincus, Academic Press, New York, 1965, pp. 411—494.
4) P. Millburn, "Metabolic Conjugation and Metabolic Hydrolysis," Vol. II, ed. by W.H. Fishman, Academic Press, New York, 1970, pp. 45—50.
5) S. Honma and T. Nambara, *Chem. Pharm. Bull.* (Tokyo), 22, 687 (1974).
6) O. Jänne, R. Vihko, J. Sjövall, and K. Sjövall, *Clin. Chim. Acta*, 23, 405 (1969); T. Cronholm, H. Eriksson, and J.-A. Gustafsson, *Eur. J. Biochem.*, 19, 424 (1971).

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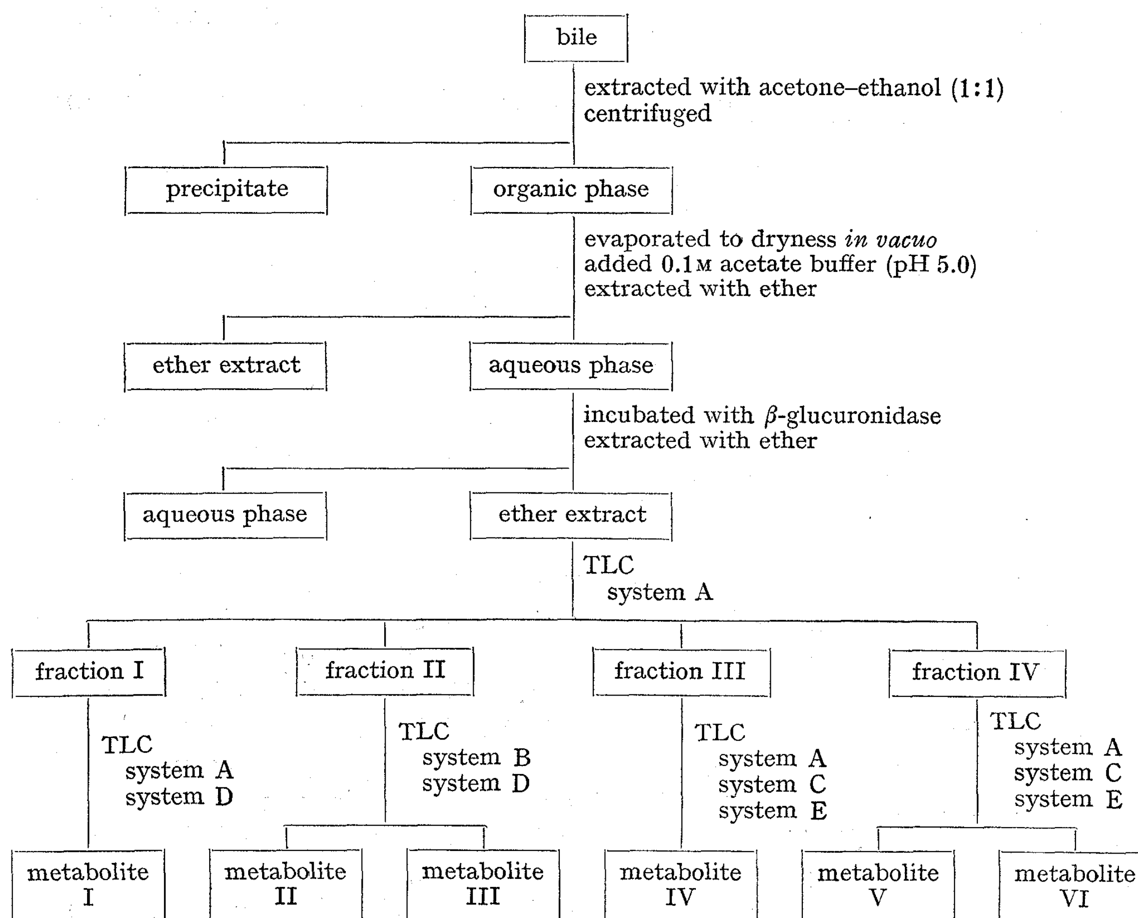

Chart 1. Separation of Biliary Metabolites in the Rat administered with Estriol

likely that metabolite I would be estriol according to TLC with system B by which four possible triols could be distinctly separated. Indeed metabolite I was identified as unchanged estriol by usual criteria.

Fraction II showed apparently a single spot on TLC with system B. On multiple runs with system D, however, the eluate was separated into two metabolites, II and III. Both the metabolites exhibited the similar mass spectra with a parent ion peak at *m/e* 318 and several fragment peaks characteristic to the phenolic steroid. These two also beared a striking resemblance in the nuclear magnetic resonance (NMR) spectra with a slight difference in the aromatic proton region. The results strongly implied that these metabolites would be isomeric 2-hydroxyestriol 2- and 3-methyl ethers. It is sufficiently substantiated that the catechol estrogen monomethyl ethers can be easily distinguished each other from the difference value in the chemical shifts of two aromatic protons.[7,8] In actuality the less polar metabolite III could be unambiguously identified as 2-methoxyestriol and, in consequence, the metabolite II as the isomeric 3-methyl ether by comparison with the authentic samples.[9]

Elution of the adsorbent corresponding to the third spot in system A and recrystallization of the eluate furnished metabolite IV. The infrared (IR) spectrum revealed the presence of the five-membered ring ketone and hydroxyl groups. With regard to the mass spectrum a molecular ion peak appeared at *m/e* 286, accompanied with the characteristic peaks due to the aromatic ring. It was hereby observed that a fragment peak of *m/e* 213 assignable to the structure missing ring D was more abundant than that of *m/e* 214. These data were indicative

7) J. Fishman and J.S. Liang, *Tetrahedron*, **24**, 2199 (1968).
8) T. Nambara, S. Honma, and S. Akiyama, *Chem. Pharm. Bull.* (Tokyo), **18**, 474 (1970).
9) J. Fishman, M. Tomasz, and R. Lehman, *J. Org. Chem.*, **25**, 585 (1960).

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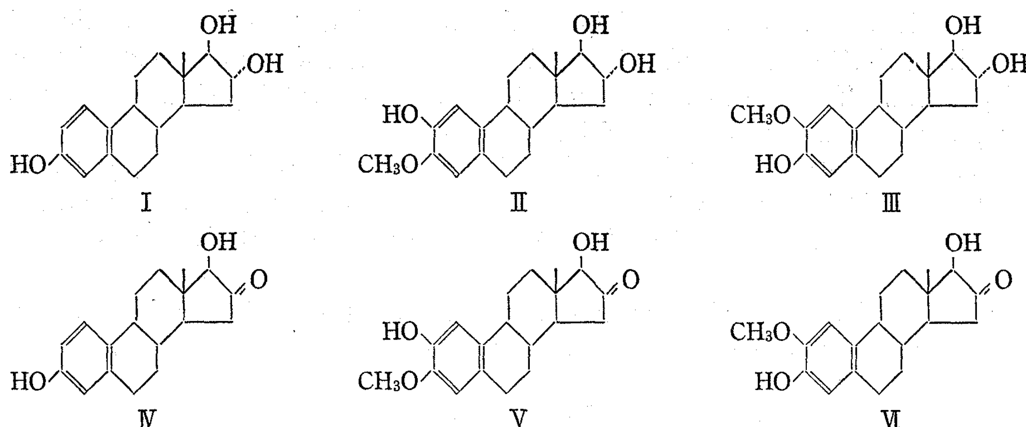

Chart 2. Metabolites excreted in Rat Bile following Administration of Estriol

of the ring D structure being the 16-oxo-17β-ol. Actually identity of the metabolite IV with synthetic 16-oxoestradiol was demonstrated by mixed melting point measurement, spectral and chromatographic comparison.

The most nonpolar fraction exhibited a single spot on TLC with system A and C. This fraction, however, could be separated into metabolite V and VI by the multiple development method using system E as a solvent. Both the metabolites exhibited a parent ion peak at *m/e* 316 and a fragmentation pattern characteristic to the aromatic steroid. The fragment peak of *m/e* 243 was found to be higher in the relative intensity than that of *m/e* 244 indicating the 16-oxo-17β-ol structure. These two also showed the similar NMR spectra with a slight difference in the aromatic proton region. The present findings led us to assume that the metabolite V and VI would be 2- and 3-monomethyl ethers of 2-hydroxy-16-oxoestradiol. Differentiation of the positional isomers was readily attained from the NMR spectral data on the aromatic protons. These evidences together permitted us to conclude that the less polar metabolite VI should be 2-methoxy-16-oxoestradiol and hence the metabolite V should be the isomeric 3-methyl ether. The assignment was unequivocally justified by direct comparison with the synthetic samples.[10]

Quantitation of the principal metabolites excreted in bile after oral administration of estriol-6,7-^{3}H was then carried out. The bile specimen collected for following 6 hr was processed and the glucuronide fraction was separated by preparative TLC in the manner as described above. It was found that 16-oxoestradiol was the most principal metabolite representing 50% of the radioactivity of the biliary glucuronides, and 2-hydroxy-16-oxoestradiol methyl ethers and 2-hydroxyestriol methyl ethers constituted 27% and 8%, respectively.

Discussion

It is first to be noted that biliary excretion of two isomeric 2-hydroxy-16-oxoestradiol methyl ethers in the rat has been demonstrated. Separation of these metabolites was performed with caution, so that the ketol rearrangement[11] did not take place during the processing. This appears to be the first record of naturally occurring catechol estrogen with the 16-oxo-17β-ol structure, although we have previously reported occurrence of two isomeric 2,16α-dihydroxyestrone 2- and 3-methyl ethers in rat bile after administration of estrone.[5]

Secondly, it should be emphasized that two pairs of the isomeric catechol estrogen monomethyl ethers were excreted in rat bile. This result is in sharp contrast with the fact that

10) T. Nambara, Y. Kawarada, M. Asama, S. Akiyama, M. Nokubo, and S. Honma, *Chem. Pharm. Bull.* (Tokyo), **21**, 2725 (1973).
11) The 16-oxo-17β-ol is most stable of four isomeric 16,17-ketols and possibly formed from the others on treatment with acid or base.[12]
12) J. Fishman, *J. Am. Chem. Soc.*, **82**, 6143 (1960).

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in human 2-methoxyestrogens are solely produced *in vivo*.[13] Selective conjugation with glucuronic acid at C-2[14,15] may probably participate in formation of a considerable amount of the 3-methyl ether. The metabolism of catechol estrogen involving O-methylation and conjugation appears to be an attractive problem to be solved.

With regard to biotransformation of ring D the 16α,17β-glycol was solely converted into the 16-oxo-17β-ol. It is of interest that there could not be seen any other modifications in ring D than dehydrogenation at C-16,[16] although the biochemical significance still remains unclear.

It is hoped that further work in progress in these laboratories will provide the more precise knowledges to interpret the characteristic nature of estrogen metabolism.

Experimental

Animals——Female Wistar rats of 9 to 10 weeks of age weighing 180 to 200 g were used. The rat was anesthesized with ether, cannulated to the bile duct with polyethylene tube by surgical operation, and housed in Bollman cage for collection of bile.

Material——Estriol-6,7-^{3}H(specific activity 5.1 Ci/mmole) was purchased from the Radiochemical Centre (Amersham, England). The radiochemical purity was *ca.* 98% according to TLC. Homogeneity of nonlabeled estriol was confirmed by TLC and melting point determination.

Administration of Estriol——All animals were starved overnight prior to administration. A suspension of estriol (40 mg) with 0.5% CMC in saline (0.5 ml) was orally given to each of fifty rats and bile was collected for following 6 hr.

Separation of Metabolites——Pooled bile (250 ml) was combined and extracted with ten-fold volume of acetone–EtOH (1:1) at 39° for 24 hr. The resulting precipitate was removed by centrifugation and washed with acetone–EtOH (1:1). The supernatant and washings were combined and evaporated *in vacuo* below 40°. To the residue was added 0.1 M acetate buffer (pH 5.0) and extracted with ether for removal of the free steroids. To the remaining aq. phase was added beef-liver β-glucuronidase (Tokyo Zōkikagaku Co.) (325 Fishman units/ml) and incubated at 37° for 96 hr. The incubation mixture was extracted with an equal volume of ether three times. After usual work-up the residue obtained was used for structural elucidation of the steroidal aglycones. Preparative TLC with system A as a solvent gave fraction I, II, III, and IV with the order of decreasing polarity. Each fraction was purified by TLC with the appropriate solvent system. Of four fractions II and IV were further separated by the multiple development method into metabolite II, III, and V, VI, respectively (see Chart 1).

Thin-Layer Chromatography (TLC)——The chromatogram was run on the plate with a layer (0.5—1 mm) of silica gel HF_{254} (E. Merck AG, Darmstadt) activated at 110° for 60 min. The following systems were used for development: system A, $CHCl_3$–EtOH (9:1); system B, AcOEt–*n*-hexane saturated with H_2O-anhydrous EtOH (80:15:5); system C, benzene–EtOH (9:1); system D, $CHCl_3$–EtOH (95:5); system E, $CHCl_3$.

Radioactivity Counting——Counting was carried out on a Aloka Model LSC-650 liquid scintillation counter. The samples were counted in a scintillator, composed of dioxane (720 ml), toluene (135 ml), MeOH (45 ml), naphthalene (100 g), 2,5-diphenyloxazole (4 g), and 1,4-bis[2-(5-phenyloxazolyl)]benzene (100 mg). The quenching was corrected by the channel ratio and external standard method.

Identification of Metabolites[17]

Estriol (Metabolite I)——Recrystallization from 90% EtOH gave metabolite I (*ca.* 15 mg) as colorless needles. mp 282—285°. Mass Spectrum *m/e*: 288 (M^+), 213, 185, 172, 159, 146. Mixed melting point on admixture with the authentic sample showed no depression, and spectral and chromatographic properties of the two samples were entirely identical.

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13) S. Kraychy and T.F. Gallagher, *J. Am. Chem. Soc.*, 79, 754 (1957).
14) K.I.H. Williams, *Steroids*, **15**, 105 (1970).
15) H. Watanabe, P. Toft, and J.S. Menzies, *J. Steroid Biochem.*, 3, 43 (1972).
16) R.I. Dorfman and F. Unger, "Metabolism of Steroid Hormones," Academic Press, New York, 1965, pp. 289—381.
17) All melting points were taken on a micro hot-stage apparatus and are uncorrected. IR spectra were obtained on a Hitachi Model EPI-G2 spectrophotometer. NMR spectra were recorded on a Hitachi Model R-22 spectrometer at 90 MHz using tetramethylsilane as an internal standard. Abbreviation used s=singlet, d=doublet, and m=multiplet. Mass spectra were run on a Hitachi Model RM-50GC under the following conditions: chamber heater 217°, sample heater 180—200°, total emission 80 μA, and accelerating voltage 1.5 kV.

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2-Hydroxyestriol 3-Methyl Ether (Metabolite II)——Recrystallization from MeOH gave metabolite II (*ca.* 14.7 mg) as colorless needles. mp 273—276°. Mass Spectrum *m/e*: 318 (M^+), 243, 215, 202, 189, 176. NMR (CD_3OD solution) δ: 0.76 (3H, s, 18-CH_3), 3.45 (1H, d, J=5 Hz, 17α-H), 3.78 (3H, s, 3-OCH_3), 4.05 (1H, m, 16β-H), 6.51 (1H, s, 4-H), 6.81 (1H, s, 1-H). Mixed melting point on admixture with the authentic sample showed no depression, and spectral and chromatographic properties of the two samples were entirely identical.

2-Methoxyestriol (Metabolite III)——Elution of the adsorbent corresponding to the spot gave metabolite III (*ca.* 16.5 mg) as pale yellow powder. mp 216—222°. Mass Spectrum *m/e*: 318 (M^+), 243, 215, 202, 189, 176. NMR (CD_3OD solution) δ: 0.76 (3H, s, 18-CH_3), 3.45 (1H, d, J=5 Hz, 17α-H), 3.78 (3H, s, 2-OCH_3), 4.05 (1H, m, 16β-H), 6.59 (1H, s, 4-H), 6.74 (1H, s, 1-H). Mixed melting point on admixture with the authentic sample showed no depression, and spectral and chromatographic properties of the two samples were entirely identical.

16-Oxoestradiol (Metabolite IV)——Recrystallization from 90% EtOH gave metabolite IV (*ca.* 12.9 mg) as colorless needles. mp 230—233°. Mass Spectrum *m/e*: 286 (M^+), 258, 214, 213, 199, 185, 172, 159, 146. NMR (CD_3OD solution) δ: 0.73 (3H, s, 18-CH_3), 3.82 (1H, s, 17α-H), 6.53 (1H, s, 4-H), 6.57 (1H, d, J=8 Hz, 2-H), 7.11 (1H, d, J=8 Hz, 1-H). IR ν_{max}^{KBr} cm^{-1}: 1740 (C=O), 3400 (OH). Mixed melting point on admixture with the authentic sample showed no depression, and spectral and chromatographic properties of the two samples were entirely identical.

2-Hydroxy-16-oxoestradiol 3-Methyl Ether (Metabolite V)——Recrystallization from 90% EtOH gave metabolite V (*ca.* 12.7 mg) as pale yellow prisms. mp 211—216°. Mass Spectrum *m/e*: 316 (M^+), 288, 244, 243, 229, 202, 189, 176. NMR ($CDCl_3$ solution) δ: 0.73 (3H, s, 18-CH_3), 3.84 (4H, s, 3-OCH_3 and 17α-H), 6.60 (1H, s, 4-H), 6.90 (1H, s, 1-H). IR ν_{max}^{KBr} cm^{-1}: 1740 (C=O), 3400 (OH). Mixed melting point on admixture with the authentic sample showed no depression, and spectral and chromatographic properties of the two samples were entirely identical.

2-Methoxy-16-oxoestradiol (Metabolite VI)——Recrystallization from 90% EtOH gave metabolite VI (*ca.* 27.2 mg) as pale yellow prisms. mp 217—220°. Mass Spectrum *m/e*: 316 (M^+), 288, 244, 243, 229, 202, 189, 176. NMR ($CDCl_3$ solution) δ: 0.73 (3H, s, 18-CH_3), 3.84 (4H, s, 2-OCH_3 and 17α-H), 6.68 (1H, s, 4-H), 6.82 (1H, s, 1-H). IR ν_{max}^{KBr} cm^{-1}: 1740 (C=O), 3400 (OH). Spectral and chromatographic comparison with the authentic sample showed identity of the two samples in every respect.

Quantitation Study——A single dose of estriol-6,7-^3H (8 μg, 3 μCi/head) was orally given to three bile fistula rats and bile was collected for 6 hr after administration. Pooled bile was combined and processed in the manner as described above. The glucuronide fraction was found to constitute 66.4% of the biliary radioactivity, whereas the free steroid only 2.9%. After addition of the authentic specimens a mixture of the steroidal aglycones derived from the glucuronide fraction was submitted to preparative TLC employing system A as a solvent. The adsorbent corresponding to each of four fractions was eluted with MeOH and the radioactivity of the eluate was counted.

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