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Conjugated Metabolites of Estriol in Rat Bile

The conjugated biliary metabolites formed from estriol have been investigated in the rat. The principal conjugates (I-XI) were separated from bile collected following administration of estriol. The structures of these metabolites were deduced from the physicochemical data and definitely characterized by direct comparison with the synthetic specimens. The significance of biotransformation hereby observed has been discussed.

A particular interest in the enterohepatic circulation of estrogen prompted us further to explore the relationship between metabolic conjugation and biliary excretion.[1] The present paper deals with the isolation and characterization of the conjugated metabolites excreted in rat bile following administration of estriol.

A suspension of estriol (50 mg) in Tween 80 was orally given to each of twenty male rats (Wistar strain, body weight 250—270 g) with cannulation to the bile duct. The pooled bile was combined and percolated through a column of Amberlite XAD-2 resin.[2,3] After washing with distilled water the steroid conjugates were eluted with 60% MeOH. The eluate was separated by partition chromatography on silica gel H (E. Merck AG) with the system $CHCl_3$–MeOH–H_2O (70: 30: 6) into six fractions. Further purification of each fraction by gel filtration on Sephadex LH-20 with H_2O as solvent provided eleven estrogen conjugates. These metabolites are numerically designated according to the order of increasing polarity.

The most nonpolar metabolite I, mp 252—255° (decomp), was isolated as colorless needles (from H_2O). This substance gave a positive Folin-Ciocalteu test and resisted to hydrolysis with beef-liver β-glucuronidase. Nuclear magnetic resonance (NMR) spectrum (D_2O solution) δ: 0.76 (3H, s, 18-CH_3), 3.39 (1H, d, J=7 Hz, 17α-H), 4.13 (1H, m, 16α-H), 6.63 (1H, s, 4-H), 7.15 (1H, s, 1-H) and infrared (IR) absorption at 1052 cm^{-1} (SO_2), together with a positive Ba^{2+}-rhodizonate test were indicative of 2-hydroxy-16-epiestriol monosulfate. Elucidation of the conjugated position was undertaken by the degradative means. Treatment with diazomethane and subsequent acid hydrolysis furnished 2-hydroxy-16-epiestriol 3-methyl ether, which proved to be identical with the authentic specimen[4] in every respect. It is evident from these data that metabolite was 2-hydroxy-16-epiestriol 2-sulfate.

Metabolite II was separated as a colorless amorphous substance. This metabolite showed the positive results with both Folin-Ciocalteu and Ba^{2+}-rhodizonate tests. Spectral data, NMR (D_2O solution) δ: 0.71 (3H, s, 18-CH_3), 3.46 (1H, d, J=6 Hz, 17α-H), 4.06 (1H, m, 16β-H), 6.63 (1H, s, 4-H), 7.16 (1H, s, 1-H) and IR ν_{max}^{KBr} cm^{-1}: 1042 (SO_2), suggested the structure of 2-hydroxyestriol monosulfate. The attached position of sulfuric acid was elucidated in the similar fashion and the resultant 2-hydroxyestriol 3-methyl ether was unambiguously characterized by direct comparison with the synthetic sample.[5] These evidences led us to assign the structure 2-hydroxyestriol 2-sulfate to metabolite II.

Unfortunately, metabolite VI could not be isolated in the crystalline state. This substance exhibited the negative test with Folin-Ciocalteu reagent and the positive with naphthoresorcinol. Enzymatic hydrolysis with β-glucuronidase yielded 2-methoxy-16-epiestriol.[4] Methylation with diazomethane, followed by usual acetylation afforded the acetate-methyl ester of 2-methoxy-16-epiestriol 3-glucuronide, which proved to be identical with the synthetic

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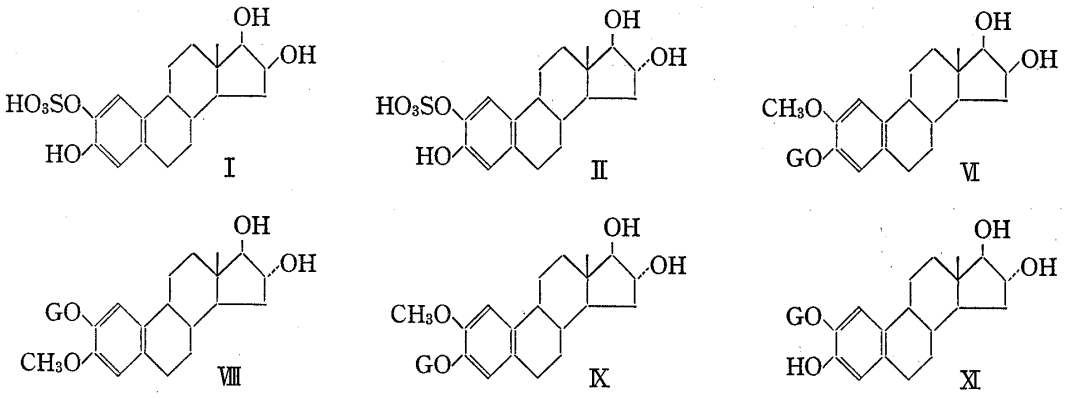
3) T. Nambara, Y.H. Bae, T. Anjyo, and S. Goya, *J. Chromatog.*, **62**, 369 (1971).
4) T. Nambara and Y. Kawarada, *Chem. Pharm. Bull.* (Tokyo), in press.
5) J. Fishman, M. Tomasz, and R. Lehman, *J. Org. Chem.*, **25**, 585 (1960).



G=glucuronyl

sample.[6] Thus the structure of 2-methoxy-16-epiestriol 3-glucuronide was unequivocally assignable to VI.

Metabolite VIII and IX showed the similar chromatographic behaviors, but could be separated with success by partition chromatography on silica gel H. Both the metabolites exhibited the negative result with Folin-Ciocalteu reagent and the positive with naphthoresorcinol. The close similarity in the NMR spectra except the aromatic ring proton signals (VIII: 6.74, 7.10; IX: 6.92 ppm) strongly indicated that these two would be isomeric each other. In actuality, hydrolysis with β-glucuronidase yielded 2-hydroxyestriol 3- and 2-monomethyl ethers. Transformation of the metabolites into the acetate-methyl esters and direct comparison with the authentic specimens[6] revealed that VIII and IX were 2-hydroxyestriol 3-methyl ether 2-glucuronide and 2-methoxyestriol 3-glucuronide, respectively.

The most polar metabolite XI was aobtained as a colorless amorphous substance. This metabolite exhibited the positive test with both Folin-Ciocalteu reagent and naphthoresorcinol. Inspection of the NMR spectrum (D_2O solution) δ: 0.72 (3H, s, 18-CH_3), 3.49 (1H, d, J=6 Hz, 17α-H), 3.50—3.96 (4H, m, pyranose-C<u>H</u>–OH, -5-H), 4.10 (1H, m, 16β-H), 4.92 (1H, d, J= 7 Hz, pyranose-1-H), 6.66 (1H, s, 4-H), 7.05 (1H, s, 1-H) suggested the structure of 2-hydroxyestriol monoglucuronide. The attached position of the sugar moiety to the steroid nucleus was determined by transforming into the known derivative. Methylation with diazomethane, followed by usual acetylation gave the acetate-methyl ester as colorless needles (from acetone-hexane), mp 187—189°, which proved to be identical with methyl (3-methoxy-16α,17β-diacetoxyestra-1,3,5(10)-trien-2-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate derived from VIII. These results lent a support to assign 2-hydroxyestriol 2-glucuronide to metabolite XI.

In addition, the remaining metabolites were similarly identified as estriol 3-, 16-, and 17-glucuronide (X, III, IV), 16-oxoestradiol 3-glucuronide (V), and 16-epiestriol 3-glucuronide (VII), respectively.

To the best of our knowledge this is the first demonstration of the occurrence of a catechol estrogen monosulfate in the living animals. In spite of much efforts, however, no evidence for the existence of the isomeric 3-sulfate in rat bile could be obtained. The present results together with the previous findings arrive at the assumption that the catechol estrogen 3-sulfate may undergo O-methylation at the unoccupied phenolic group[7,8] and subsequent transconjugation to yield 2-methoxyestrogen 3-glucuronide as a final product. It is also to be noted that catechol estrogen conjugated with glucuronic acid exclusively at the hydroxyl group in ring A. It seems very likely that hydroxylation at C-2 and conjugation with the

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7) M. Miyazaki, I. Yoshizawa, and J. Fishman, *Biochemistrv*, 8, 1669 (1969); J. Fishman, I. Yoshizawa, and L. Hellman, *Steroids*, 22, 401 (1973).
8) T. Nambara, S. Honma, and K. Kanayama, *Chem. Fharm. Bull.* (Tokyo), 20, 2235 (1972).

phenolic group may be associated with facile excretion of the metabolites into bile.

Studies on the isolation and characterization of the biliary metabolites formed from estriol are being conducted in these laboratories and the details will be reported in the near future.

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