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

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In connection with the studies on the enterohepatic circulation of estrogen the biliary metabolites formed from estrone have been investigated in the rat. The free and conjugated metabolites were separated from the bile collected after administration of estrone. The structures of these metabolites were deduced from the physico-chemical data and definitely characterized by direct comparison with the authentic specimens. The significance of the biotransformation hereby observed has been discussed.

The metabolism of estrogen is characteristic of enterohepatic circulation, which is distinctly different from that of other steroid hormones.[3] The physiological significance of this metabolic feature, however, has not yet fully been elucidated. A particular interest in these respects prompted us to explore the biliary excretion mechanism of estrogen, in which biotransformation may probably be involved. The present paper deals with separation and identification of the biliary metabolites in the rat administered with estrone.

Estrone-4-^{14}C was administered intravenously and orally to the rats with cannulation of the bile duct and the bile was collected for following 48 hr. The cumulative excretion curves of the radioactive metabolites in the bile after administration of estrone are shown in Fig. 1. The pooled bile was percolated through a column packed with Amberlite XAD-2 resin.[4] After thorough washing with distilled water the radioactive substances were eluted with methanol. The effluent was divided into two portions, one of which was used for structural elucidation of the steroidal moiety of the metabolites.

The eluate was redissolved in aqueous methanol and chromatographed on Sephadex LH-20. The conjugated metabolites thus obtained were subjected to enzymatic hydrolysis and then to solvolysis. The liberated aglycones were efficiently separated by gradient elution chromatography with the system isooctane-ethyl acetate on ethylene glycol-impregnated Celite[5] into six fractions from A to F with the order of increasing polarity as illustrated in Fig. 2.

Repeated fractional crystallization of fraction A provided metabolite I and II, whose structures were readily deduced to be 2-hydroxyestrone 2- and 3-monomethyl ethers on the basis of nuclear magnetic resonance (NMR)[6,7] and mass spectral data. These metabolites were definitely characterized by direct comparison with the authentic samples. Gas chromatographic determination[8] revealed that two isomers were formed in a ratio of *ca.* 5 to 1.

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1) This paper constitutes Part LXVIII of the series entitled "Analytical Chemical Studies on Steroids": Part LXVII: T. Nambara, Y. Kawarada, M. Asama, S. Akiyama, M. Nokubo, and S. Honma, *Chem. Pharm. Bull.* (Tokyo), **21**, 2725 (1973). A part of this work has been presented as the preliminary accounts: S. Honma and T. Nambara, *Chem. Pharm. Bull.* (Tokyo), **20**, 1343 (1972); T. Nambara, S. Honma, M. Asama, S. Akiyama, and M. Nokubo, *ibid.*, **21**, 914 (1973).

2) Location: *Aobayama, Sendai*; *a*) Present address: *Teikoku Hormone Mfg. Co., Shimosakunobe, Kawasaki*; *b*) To whom any inquiries should be addressed.

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4) H.L. Bradlow, *Steroids*, **11**, 265 (1968).

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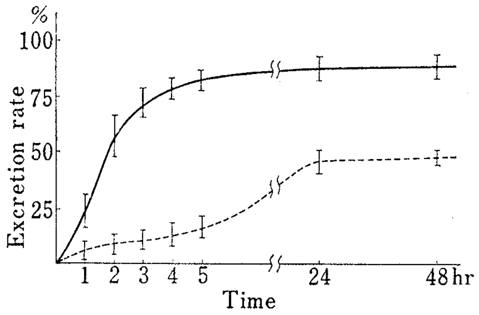

Fig. 1. Cumulative Biliary Excretion Rate of Radioactivity following Administration of Estrone-4-^{14}C

—— : *i. v.*
…… : *p. o.*

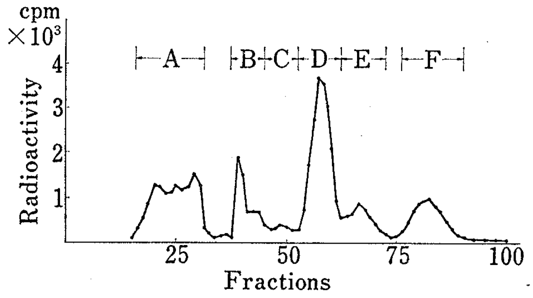

Fig. 2. Separation of Biliary Metabolites derived from Estrone-4-^{14}C by Gradient Elution Chromatography

support, Celite 545; stationary phase, ethylene glycol; mobile phase, isooctane–ethyl acetate; each fraction, 12 ml.

Purification of fraction B and C afforded metabolite III and IV, which were easily identified as unchanged estrone and estradiol, respectively.

Fraction D was further purified by preparative thin–layer chromatography (TLC). Elution of the radioactive spot gave a mixture of two metabolites, whose separation could be attained with success by multiple runs with chloroform in TLC. Recrystallization of the less polar one from benzene gave metabolite V as colorless needles. Unfortunately the more polar metabolite VI could not be isolated in the crystalline state and therefore a portion was transformed into the diacetate. Both metabolites gave the positive tetrazolium blue test, and exhibited the similar infrared (IR) spectra with an absorption band due to a five-membered ring ketone and mass spectra with a molecular ion peak at *m/e* 316. These two also beared a striking resemblance in the NMR spectra except the chemical shift of aromatic protons. The results indicated that the metabolites would be isomeric 2,16α-dihydroxyestrone 2- and 3-monomethyl ethers.[9] In actuality metabolite V could be evidently assigned to 2-methoxy-16α-hydroxyestrone by comparison with the synthetic sample.[10] The identity of metabolite VI and its 2,16-diacetate with the corresponding authentic samples was also rationalized by the usual criteria.[9,10]

Elution of the remaining radioactive spot derived from fraction D and recrystallization of the eluate furnished metabolite VII as colorless needles. The NMR and mass spectral data supported the structural assignment of 2-hydroxyestrone. Indeed the metabolite proved to be identical with the authentic specimen in every respect.

Separation of fraction E by preparative TLC afforded two phenolic substances, metabolite VIII and IX. These metabolites were converted into the crystalline acetates (VIII', IX') by usual acetylation. In the NMR spectrum VIII' exhibited a triplet signal at 5.45 ppm with a coupling constant of 5 Hz indicating the 16α-hydroxy-17-ketone structure. With regard to the mass spectrum VIII' showed fragment ions at *m/e* 172, 159, and 146, whose pattern was similar to that of estrone. It was evident from these data that metabolite VIII should be 16α-hydroxyestrone. As for the structure of another acetate (IX') the NMR signals assignable to the 18-methyl, 2-methoxyl, and two aromatic protons appeared at 0.85, 3.85, 6.70, and 6.85 ppm, respectively. In the mass spectrum a molecular ion peak occurred at *m/e* 444 accompanied with the fragment ion peaks at *m/e* 244 and 202. These findings strongly suggested that metabolite IX would be 2-methoxyestriol. The identity of metabolite IX with the synthetic sample was definitely established by direct comparison.

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10) T. Nambara, Y. Kawarada, M. Asama, S. Akiyama, M. Nokubo, and S. Honma, *Chem. Pharm. Bull.* (Tokyo), **21**, 2725 (1973).

The remaining metabolite X obtained from fraction F showed the positive Folin–Ciocalteu test. Unfortunately this substance could not be obtained crystalline and hence was led to the triacetate (X') in the usual manner. The mass and NMR spectra exhibiting a molecular ion peak at *m/e* 414 and aromatic proton signals at 6.85 and 7.01 ppm permitted the assignment of 2-hydroxyestradiol triacetate to X'. As was expected the mixed melting point measurement and spectral comparison with the synthetic specimen justified this assumption. The structure 2-hydroxyestradiol was thus unequivocally assigned to metabolite X.

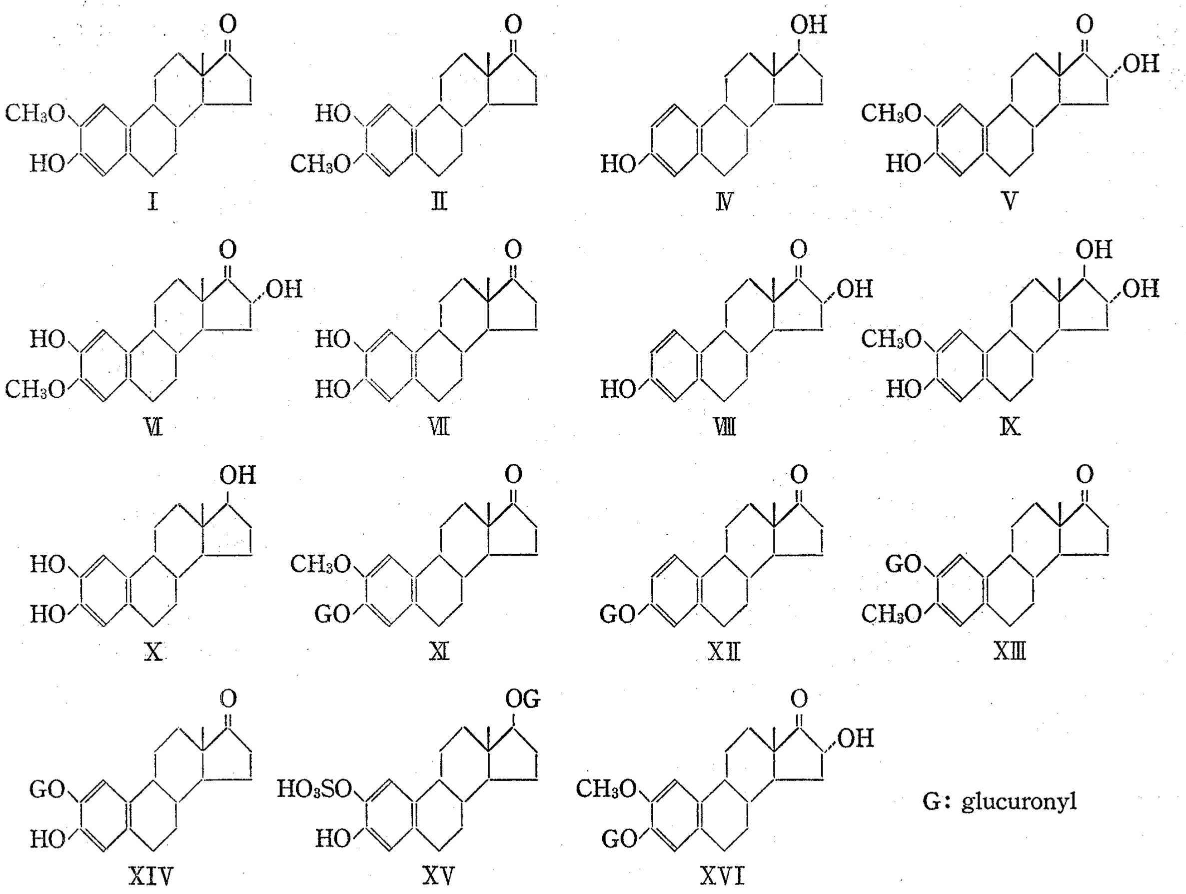

Chart 1. Metabolites excreted in Rat Bile following Administration of Estrone

Next effort was directed to separation and characterization of the conjugated metabolites. The remaining eluate obtained by Amberlite XAD-2 resin chromatography was subjected to gel filtration on Sephadex G-25 to give two fractions, A and B. Further purification of fraction A by chromatography on Sephadex LH-20, followed by recrystallization furnished metabolite XI. This substance showed the positive reaction with naphthoresorcinol and underwent readily hydrolysis with β-glucuronidase to yield 2-methoxyestrone. Treatment with diazomethane and then with acetic anhydride and pyridine afforded the acetate-methyl ester (XI'). In the NMR spectrum an anomeric proton signal appeared at 5.05 ppm with a coupling constant of 8 Hz indicating the β-glucoside linkage. With regard to the mass spectrum parent ion appeared at *m/e* 616 and fragment ions assignable to the sugar and steroidal moieties at *m/e* 317 and 300, respectively. On the basis of these evidences XI' was assignable to the structure methyl (2-methoxy-17-oxoestra-1,3,5(10)-trien-3-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate. In addition the spectral properties and chromatographic behaviors of metabolite XI proved to be entirely identical with those of authentic 2-methoxyestrone 3-glucuronide.[11]

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[11] S. Honma and T. Nambara, *Chem. Pharm. Bull.* (Tokyo), **20**, 1343 (1972).

From the mother liquor of metabolite XI the second conjugate, metabolite XII, was separated as amorphous substance. Derivatization in the usual manner yielded the acetate-methyl ester (XII'), which exhibited molecular ion at *m/e* 586 and fragment ions due to the glucuronyl and steroidal residues at *m/e* 317 and 270. It was assumed from these data that metabolite XII would be estrone 3-glucuronide. Actually, direct comparison with the synthetic sample justified the structural assignment.

A portion of the mother liquor was hydrolyzed with β-glucuronidase to give catechol estrogen monomethyl ether. Difficulties were encountered with isolation of the conjugate and therefore the crude metabolite was submitted to usual acetylation. Repeated fractional crystallization of the product gave the desired acetate-methyl ester (XIII') as colorless needles. Upon acid hydrolysis under the mild conditions XIII' yielded 2-hydroxyestrone 3-methyl ether. The occurrence of the peaks assignable to molecular ion (*m/e* 616) and fragment ions (*m/e* 317 and 300) lent a support to assign the structure methyl (3-methoxy-17-oxoestra-1,3,5(10)-trien-2-yl-2,3,4-tri-O-acetyl-β-D-glucopyranoside)uronate to XIII'. The acetate-methyl ester was prepared from 2-hydroxyestrone 3-methyl ether and acetobromoglucuronate by Koenigs–Knorr reaction. The identity of XIII' with this synthetic sample was demonstrated by mixed melting point measurement and spectral comparison. These results led us to conclude that metabolite XIII should be 2-hydroxyestrone 3-methyl ether 2-glucuronide.

The remaining fraction B was separated into two radioactive metabolites by paper chromatography. The less polar one was further purified by gel filtration on LH-20 yielding metabolite XIV as colorless prisms. This substance showed the positive reactions with both naphthoresorcinol and Folin–Ciocalteu reagent and underwent enzymatic hydrolysis to afford 2-hydroxyestrone. In order to determine the attached position of a glucuronyl residue to the steroid nucleus, metabolite XIV was treated with diazomethane and then with acetic anhydride and pyridine. Hydrolytic cleavage of a glucuronoside linkage in the resulting acetate-methyl ester (XIV') with mineral acid furnished 2-hydroxyestrone 3-methyl ether. Based upon these facts metabolite XIV was definitely characterized as 2-hydroxyestrone 2-glucuronide.

The more polar fraction described above was similarly purified by chromatography on Sephadex LH-20 and then on alumina to provide metabolite XV. This substance showed the positive naphthoresorcinol test and the IR absorption bands due to a sulfonate group at 1040 and 1150 cm^{-1}. Upon treatment with mineral acid under mild conditions metabolite XV underwent hydrolysis to yield 2-hydroxyestradiol. With regard to the NMR spectrum the chemical shift of 18-methyl proton (0.88 ppm) was more analogous to that of the 17β-glucuronide rather than that of the 17β-sulfate. These data indicated that sulfonic acid would be bound to either of two phenolic groups in ring A. For the purpose of determining this point metabolite XV was treated with diazomethane and the product was in turn hydrolyzed with mineral acid. Hereupon, formation of 2-hydroxyestradiol 3-methyl ether was demonstrated by gas chromatography-mass spectrometry (GC-MS). These results led us to assign 2-hydroxyestradiol 2-sulfate 17-glucuronide to metabolite XV.

A portion of the eluate obtained by Sephadex G-25 chromatography was purified repeatedly on Sephadex LH-20 to provide metabolite XVI. This conjugate gave the positive reactions with both naphthoresorcinol and tetrazolium blue but the negative with Folin–Ciocalteu reagent. In the NMR spectrum the 18-methyl and methoxyl proton signals appeared at 0.97 and 3.80 ppm, respectively. Hydrolysis with β-glucuronidase yielded 2-methoxy-16α-hydroxyestrone, which was unequivocally identified by GC-MS. In addition metabolite XVI was led to the acetate-methyl ester (XVI'), which exhibited the characteristic fragment peaks at *m/e* 358, 317, 202, and 155. Upon comparison with the synthetic sample[10] XVI' proved to be the 3-glucuronide acetate-methyl ester. These findings together permitted us to assign the structure 2-methoxy-16α-hydroxyestrone 3-glucuronide to metabolite XVI.

Discussion

The cumulative excretion rates of the biliary metabolites for 24 hr following the intravenous and oral administration of radioactive estrone were found to be *ca.* 90% and 50%, respectively. It seems likely that biliary excretion of estrogen is much more significant in the rat than in human[12] and other species.

The principal metabolites excreted in the rat bile has proved to be catechol estrogens and their monomethyl ethers. It is first to be noted that *in vivo* O-methylation occurred at the catechol group to yield the 2- and 3-monomethyl ethers in a ratio of *ca.* 5 to 1. Recently, excretion of 2-hydroxyestrone 3-methyl ether in the rat bile and human pregnancy urine has been demonstrated.[13,14] Although the definite evidence is still unavailable, formation of the 2-monomethyl ether in much larger amounts is ascribable to the participation of selective conjugation. The previous works indicated that the sulfate formation would be involved in directive O-methylation to a greater extent than glucuronidation.[15,16]

It is also noteworthy that two isomeric monomethyl ethers of 2,16α-dihydroxyestrone have been unequivocally identified. To the best of our knowledge this is the first recorded instance of naturally occurring catechol estrogen with the 16,17-ketol structure.[17] The attached position of a glucuronyl moiety in metabolite XVI has proved to be exclusively C-3. This is in contrast with an account that 16α-hydroxyestrone was also excreted as the 16-glucuronide in human pregnancy urine.[19]

In recent years Layne and his co-workers disclosed the occurrence of the novel conjugates of estrogen linked with N-acetylglucosamine[20] and glucose.[21] With respects to these findings the conjugated metabolites have been characterized by leading to the acetate-methyl ester and direct comparison with the synthetic specimen. Although two phenolic groups of catechol estrogen are chemically indistinguishable, glucuronidation takes place selectively at C-2 in the rat,[22,23] hamster,[23] and guinea pig,[24] while at C-3 in human.[25] The species difference in the mode of conjugation is of particular interest in respect of multiplicity and specificity of this enzyme. In this study, however, *in vivo* formation of 2-methoxyestrone 3-glucuronide has been clarified. The metabolism of catechol estrogen involving O-methylation and glucuronidation appears to be an attractive problem to be solved.

It should be also emphasized that a new double conjugate, 2-hydroxyestradiol 2-sulfate 17-glucuronide, has been separated and characterized. So far as we know this is the second example of the estrogen sulfate-glucuronide as a biliary metabolite since the first record by Levitz, *et al.*[26] dealing with excretion of estriol 3-sulfate 16-glucuronide in human bile.

It has already been demonstrated that incubation with the rat liver slices formed the sulfate and glucuronide in approximately equivalent amounts.[27] In the present case any

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[17] After contribution of our preliminary note,[9] we learned a paper dealing with biliary excretion of 2-methoxy-16α-hydroxyestrone in the rat.[18]

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appreciable amount of the estrogen sulfate was not excreted in the bile. These results imply that conjugation with glucuronic acid may probably favor biliary excretion and hence enterohepatic circulation. However, the biochemical significance of C-2 hydroxylation in biliary excretion still remains unclear. It is hoped that further work in progress in these laboratories will provide the data necessary to explain the characteristic nature of estrogen metabolism.

Experimental

Animal——Male Wistar rats weighing, on the average, 350 g were used. The rat was anesthesized with sodium pentobarbital, cannulated to the bile duct with polyethylene tube (PE 50, Clay Adams, Parsippany, N.J.) by surgical operation, and housed in Bollman cage for collection of the bile.

Material——Estrone-4-^{14}C was purchased from Daiichi Pure Chemicals Co. (Tokyo) and the radiochemical purity was checked by TLC. Amberlite XAD-2 resin (Rohm and Haas Co., Philadelphia), Sephadex G-25 and LH-20 (Pharmacia Fine Chemicals, Uppsala) were purchased.

Radioactivity Counting——Counting was carried out on a Kobe Kogyo Model EA-118 liquid scintillation counter. For the toluene-soluble samples toluene containing 2,5-diphenyloxazole (4 g/liter) and 1,4-bis[2-(5-phenyloxazolyl)]benzene (400 mg/liter) was used as a scintillator. The aqueous samples were counted in a scintillator, composed of dioxane (1 liter), naphthalene (100 g), 2,5-diphenyloxazole (4 g), and 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (100 mg). The quenching was corrected by the channel ratio and external standard methods.

Paper Chromatography (PC)——Whatman No. 3 filter paper was used for decending chromatography. Development was carried out employing the system *sec*-BuOH–H_2O (1:1) after tank equilibration.

Thin-Layer Chromatography (TLC)——The chromatogram was run on the plate of silica gel HF_{254} (E. Merck AG, Darmstadt) activated at 140° for 2.5 hr.

Gas-Liquid Chromatography (GLC)——The apparatus used was a Shimadzu Model GC-4BM gas chromatograph equipped with a hydrogen flame ionization detector and a U-shaped glass tube (3 mm i.d.) packed with 3% SE-30 on Gas Chrom P (60—80 mesh). The temperatures of column, detector, and injection chamber were kept at 230°, 260°, and 260°, respectively. N_2 was used as the carrier gas at a flow rate of 80 ml/min.

Gas Chromatography-Mass Spectrometry (GC-MS)——The apparatus used was a Hitachi Model RMU-6E spectrometer. Spectral measurement was run under the following conditions: ionization voltage 70 eV, accelerator voltage 1800 V, temperature of ionization chamber 200°, and width of collector slit 0.3 mm. He was used as the carrier gas at a flow rate of 60 ml/min.

Syntheses of Samples

Methyl (3-Methoxy-17-oxoestra-1,3,5(10)-trien-2-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (1)——To a solution of 2-hydroxyestrone 3-methyl ether (260 mg) and methyl 1-bromo-1-deoxy-2,3,4-tri-O-acetyl-α-D-glucopyranuronate (610 mg) in anhydrous benzene (8 ml) was added freshly prepared Ag_2CO_3 (1.2 g) and stirred in the dark place for 24 hr. After removal of the precipitate by filtration the filtrate was evaporated *in vacuo*. An oily residue was submitted to preparative TLC. Elution of the adsorbent corresponding to the spot and recrystallization of the eluate from MeOH gave 1 (66 mg) as colorless needles. mp 175—177°. $[\alpha]_D^{20}$ +63.0° (c=0.06, $CHCl_3$). *Anal.* Calcd. for $C_{32}H_{40}O_{12}$: C, 62.32; H, 6.54. Found: C, 62.49; H, 6.31.

Methyl (3-Benzyloxy-17-oxoestra-1,3,5(10)-trien-2-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (2)——To a solution of 2-hydroxyestrone 3-benzyl ether (1.1 g) and methyl 1-bromo-1-deoxy-2,3,4-tri-O-acetyl-α-D-glucopyranuronate (1.7 g) in anhydrous benzene (70 ml) was added freshly prepared Ag_2CO_3 (3 g) and stirred in the dark place for 24 hr. After removal of the precipitate by filtration the filtrate was evaporated *in vacuo*. An oily residue was submitted to preparative TLC. Elution of the adsorbent corresponding to the spot and recrystallization of the eluate from MeOH gave 2 (180 mg) as colorless needles. mp 220—222°. $[\alpha]_D^{21}$ +31.6° (c=0.09, $CHCl_3$). *Anal.* Calcd. for $C_{38}H_{44}O_{12}$: C, 65.88; H, 6.40. Found: C, 65.68; H, 6.18.

Methyl (3-Hydroxy-17-oxoestra-1,3,5(10)-trien-2-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (3)——A solution of 2 (90 mg) in MeOH (150 ml) was shaken with 10% Pd/C (35 mg) under a stream of H_2 gas at room temperature for 24 hr. After removal of the precipitate by filtration the filtrate was evaporated *in vacuo*. The crude product was recrystallized from MeOH to give 3 (76 mg) as colorless needles. mp 210—213°. $[\alpha]_D^{21.5}$ +53.0° (c=0.10, $CHCl_3$). *Anal.* Calcd. for $C_{31}H_{38}O_{12}$: C, 61.78; H, 6.36. Found: C, 61.80; H, 6.36.

Sodium (3-Hydroxy-17-oxoestra-1,3,5(10)-trien-2-yl-β-D-glucopyranosid)uronate (2-Hydroxyestrone 2-Glucuronide) (4)——To a solution of 3 (10 mg) in MeOH (2 ml) was added 1 N NaOH (0.11 ml) and allowed to stand at room temperature for 24 hr. The precipitate was collected by filtration and recrystallized from MeOH to give 4 (2.7 mg) as colorless plates. mp 248—252° (decomp.). *Anal.* Calcd. for $C_{24}H_{29}O_9Na \cdot 3H_2O$: C, 53.52; H, 6.55. Found: C, 53.23; H, 5.93.

Administration of Estrone——A solution of estrone-4-^{14}C (1 μmole, 1 μCi) dissolved in propylene glycol (0.2 ml)–saline (2 ml) was injected intravenously. A suspension of estrone-4-^{14}C (25 mg, 1.5 μCi) with Tween 80 in saline (2 ml) was orally given to each of four rats and the nonlabeled steroid (25 mg) was similarly administered to each of forty rats.

Separation of Metabolites——The pooled bile was combined (1500 ml) and percolated through a column packed with Amberlite XAD-2 resin (55×6 cm i.d.), washed with H_2O (2 liter) at a flow rate of 400 ml/hr and then eluted with MeOH (3 liter) at a flow rate of 1 liter/hr. One-third portion of the radioactive effluent was used for structural elucidation of the aglycones. After evaporation of solvent a gummy residue obtained was redissolved in distilled water (200 ml), adjusted to pH 4.5 with acetate buffer (50 ml), and then incubated with beef-liver β-glucuronidase (5000 Fishman units/ml) at 37° for 48 hr. The incubated fluid was extracted twice with ether, and the organic phase was washed with H_2O and dried over anhydrous Na_2SO_4. The remaining aqueous phase was brought to pH 1 with 20 N H_2SO_4 (10 ml) and extracted with AcOEt. The organic phase was combined and allowed to stand at 37° for 24 hr. The extract was washed with 5% $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4, and evaporated. The deconjugated metabolites derived from the glucuronide and sulfate fractions were separated by gradient elution chromatography with the system isooctane-ethyl acetate on ethylene glycol-impregnated Celite 545 into six fractions from A to F with the increasing polarity as follows:

Fraction	Weight (mg)	Metabolite
A	58	I, II
B	60	III
C	32	IV
D	139	V, VI, VII
E	79	VIII, IX
F	58	X

Each fraction, if necessary, was submitted to further purification by preparative TLC.

Two-third portion of the effluent obtained by chromatography on Amberlite XAD-2 resin was used for structural elucidation of the conjugated metabolites. After evaporation of solvent a gummy residue was subjected to gel filtration on Sephadex LH-20 (53×4 cm i.d.) using 50% aq. MeOH as eluent. The eluate was further divided into two fractions by gel filtration on Sephadex G-25 using 2 N NH_4OH–*n*-BuOH–*tert*-BuOH (133: 200: 200) as follows:

Fraction	Weight (g)	Metabolite
A	3.2	XI, XII, XIII
B	0.7	XIV, XV

Identification of Metabolites[28]

2-Methoxyestrone (Metabolite I)——Recrystallization of fraction A from MeOH gave metabolite I (30 mg) as colorless needles. mp 189—191°. IR $\nu_{\max}^{\mathrm{KBr}}$ cm^{-1}: 1730 (five-membered ring C=O). NMR ($CDCl_3$ solution) δ: 0.90 (3H, s, 18-CH_3), 3.85 (3H, s, 2-OCH_3), 6.65 (1H, s, aromatic 4-H), 6.80 (1H, s, aromatic 1-H). Mass Spectrum *m/e*: 300 (M^+), 285, 243, 202. Mixed melting point on admixture with the authentic sample showed no depression, and IR, NMR, and mass spectra of two samples were entirely identical.

2-Hydroxyestrone 3-Methyl Ether (Metabolite II)——The residue obtained from the mother liquor of metabolite I was recrystallized from hexane–benzene to give metabolite II (9.1 mg) as colorless needles. mp 178.5—181.5°. IR $\nu_{\max}^{\mathrm{KBr}}$ cm^{-1}: 1730 (five-membered ring C=O). NMR ($CDCl_3$ solution) δ: 0.90 (3H, s, 18-CH_3), 3.88 (3H, s, 3-OCH_3), 6.60 (1H, s, aromatic 4-H), 6.89 (1H, s, aromatic 1-H). Mass Spectrum *m/e*: 300 (M^+), 285, 243. Mixed melting point on admixture with the authentic sample showed no depression, and IR, NMR, and mass spectra of two samples were entirely identical. Inspection by GLC revealed that fraction A consisted of metabolite I and II in a ratio of 5 to 1.

Estrone (Metabolite III)——The eluate of fraction B was recrystallized from MeOH to give metabolite III (17 mg) as colorless needles. mp 246—249°. Comparison with the authentic specimen showed the identi-

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28) All melting points were taken on a micro hot-stage apparatus and are uncorrected. Infrared (IR) spectra were run on Hitachi Model EPI-G2 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded on Hitachi Model R-20A spectrometer at 60 MHz using tetramethylsilane as an internal standard. Abbreviation used s=singlet, d=doublet, t=triplet, and m=multiplet.

ty of two samples in every respect.

Estradiol (Metabolite IV)——Fraction C was further purified by preparative TLC employing benzene–MeOH (10: 1) as developing solvent. Elution of the adsorbent corresponding to the spot and recrystallization of the eluate from MeOH gave metabolite IV (1 mg) as colorless needles. mp 175—179°. Mixed melting point on admixture with the authentic sample showed no depression, and IR spectra and chromatographic behaviors of two samples were entirely identical.

2-Methoxy-16α-hydroxyestrone (Metabolite V)——Fraction D was submitted to preparative TLC employing $CHCl_3$–MeOH (97: 3) as developing solvent. Elution of the adsorbent corresponding to the spot (*Rf* 0.40) gave an amorphous substance (12 mg). Further purification by preparative TLC with $CHCl_3$ as solvent on multiple runs, followed by recrysrtallization of the less polar fraction from benzene gave metabolite V (5 mg) as colorless needles. mp 199—201.5°. This substance showed the positive reactions with both tetrazolium blue and Folin–Ciocalteu reagent. IR $\nu_{\max}^{\mathrm{KBr}}$ cm^{-1}: 3400 (OH), 1730 (five-membered ring C=O). NMR ($CDCl_3$ solution) δ: 0.98 (3H, s, 18-CH_3), 3.85 (3H, s, 2-OCH_3), 4.40 (1H, t, $J=5$ Hz, 16β-H), 6.64 (1H, s, aromatic 4-H), 6.75 (1H, s, aromatic 1-H). Mass Spectrum *m/e*: 316 (M^+), 244, 202, 176. Mixed melting point on admixture with the authentic sample[10] showed no depression, and IR, NMR, and mass spectra of two samples were entirely identical.

2,16α-Dihydroxyestrone 3-Methyl Ether (Metabolite VI)——The more polar fraction, obtainable by preparative TLC on multiple runs with $CHCl_3$ as described above, was eluted with acetone to give metabolite VI (2 mg) as colorless oil. This substance showed the positive reactions with both tetrazolium blue and Folin–Ciocalteu reagent. NMR ($CDCl_3$ solution) δ: 0.98 (3H, s, 18-CH_3), 3.85 (3H, s, 3-OCH_3), 4.40 (1H, t, $J=5$ Hz, 16β-H), 6.57 (1H, s, aromatic 4-H), 6.85 (1H, s, aromatic 1-H). Mass Spectrum *m/e*: 316 (M^+), 202. 2,16-Diacetate: Treatment with Ac_2O and pyridine in the usual manner, followed by recrystallization from acetone–hexane gave the 2,16-diacetate as colorless needles. mp 213—218°. Mass Spectrum *m/e*: 400 (M^+), 358, 316, 244. Mixed melting point on admixture with the authentics ample[10] showed no depression, and IR, NMR, and mass spectra of two samples were entirely identical.

2-Hydroxyestrone (Metabolite VII)——The eluate of the spot (*Rf* 0.25), obtainable from fraction D by preparative TLC with $CHCl_3$–MeOH (97: 3) as solvent, was recrystallized from benzene to give metabolite VII (40 mg) as colorless needles. mp 203—207°. IR $\nu_{\max}^{\mathrm{KBr}}$ cm^{-1}: 3400 (OH), 1730 (five-membered ring C=O). Mixed melting point on admixture with the authentic sample showed no depression, and IR spectra of two samples were entirely identical.

16α-Hydroxyestrone (Metabolite VIII)——Fraction E was further purified by preparative TLC employing benzene–MeOH (9: 1) as developing solvent to give metabolite VIII (7 mg) as colorless oil. This substance showed positive reactions with both tetrazolium blue and Folin–Ciocalteu reagent. Treatment with Ac_2O and pyridine in the usual manner, followed by recrystallization from MeOH gave the 3,16-diacetate (VIII') (4 mg) as colorless needles. mp 171—174°. NMR ($CDCl_3$ solution) δ: 1.00 (3H, s, 18-CH_3), 2.12, 2.28 (6H, s, 3,16α-$OCOCH_3$), 5.45 (1H, t, $J=5$ Hz, 16β-H), 6.70—7.00 (3H, m, aromatic 1,2,4-H). Mass Spectrum *m/e*: 370 (M^+), 328, 286, 268, 240, 214. Direct comparison of metabolite VIII and the 3,16-diacetate with the authentic specimens showed the identity of two samples, respectively.

2-Methoxyestriol (Metabolite IX)——Fraction E was further purified by preparative TLC employing benzene–MeOH (9: 1) as developing solvent to give metabolite IX (2 mg) as colorless oil. This substance showed the positive Folin–Ciocalteu test. Treatment with Ac_2O and pyridine in the usual manner, followed by recrystallization from MeOH gave the 3, 16,17-triacetate (IX') as colorless needles. mp 188—191°. NMR ($CDCl_3$ solution) δ: 0.85 (3H, s, 18-CH_3), 2.04, 2.08, 2.28 (9H, s, 3,16α,17β-$OCOCH_3$), 3.85 (3H, s, 2-OCH_3), 6.70 (1H, s, aromatic 4-H), 6.85 (1H, s, aromatic 1-H). Mass Spectrum *m/e*: 444 (M^+), 402, 387, 358, 342, 300. Direct comparison of metabolite IX and the 3,16,17-triacetate with the authentic specimens showed the identity of two samples, respectively.

2-Hydroxyestradiol (Metabolite X)——Fraction F was further purified by gel filtration on Sephadex LH-20 (96 × 1.8 cm i.d.) employing 80% MeOH as eluent to give metabolite X (8 mg) as colorless oil. This substance showed the positive Folin–Ciocalteu test. Treatment of metabolite X with Ac_2O and pyridine in the usual manner, followed by recrystallization from benzene–hexane gave the 2,3,17-triacetate (X') (3 mg) as colorless needles. mp 165—168°. NMR ($CDCl_3$ solution) δ: 0.80 (3H, s, 18-CH_3), 2.05, 2.25 (9H, s, 2,3,17β-$OCOCH_3$), 6.85 (1H, s, aromatic 4-H), 7.01 (1H, s, aromatic 1-H). Mass Spectrum *m/e*: 414 (M^+), 330, 270, 188. Direct comparison of metabolite X and the 2,3,17-triacetate with the authentic specimens showed the identity of two samples, respectively.

2-Methoxyestrone 3-Glucuronide (Metabolite XI)——Fraction A was submitted to gel filtration on Sephadex LH-20 (107 × 2 cm i.d.) employing 80% MeOH as eluent. Fractional crystallization of the eluate (470 mg) from MeOH–iso-PrOH gave metabolite XI (90 mg) as amorphous substance. mp 211—215°. This substance showed the positive naphthoresoroinol test. A portion of metabolite XI (2 mg) was dissolved in H_2O (3 ml) and incubated with beef-liver β-glucuronidase (30000 Fishman units) at 38° for 48 hr. The incubation mixture was extracted with ether and the organic phase was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated. The hydrolyzate thus obtained proved to be 2-methoxyestrone by direct comparison with the authentic sample. TLC (benzene–MeOH (9: 1)): *Rf* 0.80. NMR (CD_3OD solution) δ: 0.90 (3H, s, 18-CH_3), 3.82 (3H, s, 2-OCH_3), 6.90 (2H, s, aromatic 1,4-H). IR $\nu_{\max}^{\mathrm{KBr}}$ cm^{-1}: 1738 (five-membered ring C=O). To a solution of metabolite XI (10 mg) in MeOH (3 ml) was added an ethereal solution of CH_2N_2 and allowed to stand at room temperature for 30 min. After evaporation of solvent the crude product was treated with Ac_2O and pyridine in the usual manner. Recrystallization from MeOH gave the acetate–methyl ester (XI') as colorless needles. mp 214—217°. NMR ($CDCl_3$ solution) δ: 0.90 (3H, s, 18-CH_3), 2.00—2.10 (9H, m, –$OCOCH_3$), 3.72, 3.80 (6H, s, –$COOCH_3$, 2-OCH_3), 5.05 (1H, d, $J=8$ Hz, 1'-H), 6.82 (2H, s, aromatic 1, 4-H). Mass Spectrum *m/e*: 616 (M^+), 317, 300. Mixed melting point on admixture with the authentic specimen showed no depression, and IR spectra of two samples were entirely identical.

Estrone 3-Glucuronide (Metabolite XII)——The residue obtained from the mother liquor of metabolite XI was recrystallized from MeOH–iso-PrOH to give metabolite XII as amorphous substance. mp >300°. This substance showed the positive naphthoresorcinol test and underwent hydrolysis with beef-liver β-glucuronidase to give estrone. Treatment of metabolite XII with CH_2N_2 and then with Ac_2O and pyridine, followed by recrystallization from MeOH gave the acetate–methyl ester (XII') as colorless needles. mp 226—228°. NMR ($CDCl_3$ solution) δ: 0.90 (3H, s, 18-CH_3), 2.05 (9H, s, –$OCOCH_3$), 3.72 (3H, s, –$COOCH_3$), 6.60—7.20 (3H, s, aromatic H). Mass Spectrum *m/e*: 586 (M^+), 317, 270, 215. Mixed melting point on admixture with the authentic sample showed no depression, and IR spectra of two samples were entirely identical.

2-Hydroxyestrone 3-Methyl Ether 2-Glucuronide (Metabolite XIII)——The mother liquor of metabolite XI and XII was submitted to hydrolysis with beef-liver β-glucuronidase and the liberated aglycone was characterized to be 2-hydroxyestrone 3-methyl ether by TLC using $CHCl_3$ as developing solvent. Treatment of metabolite XIII with CH_2N_2 and then with Ac_2O and pyridine, followed by recrystallization from aq. MeOH gave the acetate–methyl ester (XIII') (0.5 mg) as colorless needles. mp 173—177°. Mass Spectrum *m/e*: 616 (M^+), 317, 300, 215. Mixed melting point on admixture with the authentic sample showed no depression.

2-Hydroxyestrone 2-Glucuronide (Metabolite XIV)——Fraction B was submitted to PC using *sec*-BuOH–H_2O (1: 1) as developing solvent. The eluate from the spot (*Rf* 0.59) was further purified by chromatography on Sephadex LH-20 using 60% MeOH–0.2 M AcOH as eluent. Recrystallization of the eluate from MeOH gave metabolite XIV as colorless prisms. mp 245—249° (decomp.). This substance showed the positive reactions with both naphthoresorcinol and Folin-Ciocalteu reagent. NMR (CD_3OD solution) δ: 0.90 (3H, s, 18-CH_3), 6.55 (1H, s, aromatic 4-H), 7.13 (1H, s, aromatic 1-H). IR ν_{max}^{KBr} cm^{-1}: 1730 (five-membered ring C=O), 1610 (–COOH). Treatment of metabolite XIV with CH_2N_2 for a prolonged period and then with Ac_2O and pyridine, followed by recrystallization from MeOH gave the acetate–methyl ester (XIV') as colorless needles. mp 174—177°. Mixed melting point on admixture with the authentic sample showed no depression. NMR ($CDCl_3$ solution) δ: 0.90 (3H, s, 18-CH_3), 2.01, 2.10 (9H, s, –$OCOCH_3$), 3.72, 3.75 (6H, s, –$COOCH_3$, 3-OCH_3), 6.60 (1H, s, aromatic 4-H), 7.10 (1H, s, aromatic 1-H). Mass Spectrum *m/e*: 616 (M^+), 317, 300. A portion of XIV' was dissolved in MeOH (0.5 ml)–conc. HCl (0.3 ml) and refluxed for 1 hr. The hydrolyzate was identified as 2-hydroxyestrone 3-methyl ether by TLC using $CHCl_3$ as developing solvent.

2-Hydroxyestradiol 2-Sulfate 17-Glucuronide (Metabolite XV)——Fraction B was submitted to PC with use of *sec*-BuOH–H_2O (1: 1) as solvent, and the area corresponding to the conjugate (*Rf* 0.34) was eluted with H_2O. The eluate was purified by chromatography on Sephadex LH-20 (95×2 cm i.d.) using 70% MeOH as eluant. Subsequent chromatography on neutral Al_2O_3 (20×1 cm i.d.) employing 0.5% NH_4OH/EtOH as solvent afforded colorless powder (9 mg). Further gel filtration on Sephadex LH-20 (50×1 cm i.d.) using 60% MeOH, followed by recrystallization from ether gave metabolite XV (2 mg) as amorphous substance. IR ν_{max}^{KBr} cm^{-1}: 1040, 1150, 1600. NMR (CD_3OD solution) δ: 0.88 (3H, s, 18-CH_3). To a solution of metabolite XV in MeOH was added an ethereal solution of CH_2N_2 and allowed to stand at room temperature for 48 hr. After evaporation of solvent the product was dissolved in MeOH (1 ml)–conc. HCl (0.2 ml) and heated in a sealed tube for 1 hr. After usual work-up the crude product obtained was subjected to GC-MS. Retention time: 9.0 min. Mass Spectrum *m/e*: 302 (M^+). This transformation product was thus elucidated to be 2-hydroxyestradiol 2-methyl ether. These data were evidences that metabolite XV should be 2-hydroxyestradiol 2-sulfate 17-glucuronide.

2-Methoxy-16α-hydroxyestrone 3-Glucuronide (Metabolite XVI)——The eluate obtained by gel filtration on Sephadex G-25 was further purified by Sephadex LH-20 chromatography using 60% MeOH–0.2 M AcOH as eluent to give metabolite XVI (13 mg) as amorphous substance. mp 184—190°. This substance showed the positive reactions with both tetrazolium blue and naphthoresorcinol but the negative Folin–Ciocalteu test. NMR (CD_3OD solution) δ: 0.97 (3H, s, 18-CH_3), 3.80 (3H, s, 2-OCH_3), 6.75—6.90 (2H, m, aromatic 1,4-H). A portion of metabolite XVI (1 mg) was hydrolyzed with beef-liver β-glucuronidase to give 2-methoxy-16α-hydroxyestrone, which was identified by GC-MS. Treatment of metabolite XVI with CH_2N_2 and then with Ac_2O and pyridine, followed by recrystallization from MeOH gave the acetate–methyl ester (XVI') as colorless needles. mp 105—113°. The chromatographic behaviors and IR, NMR, and mass spectra of XVI' were entirely identical with those of the authentic samples.[10]

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