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Studies on Metabolism of 3-Desoxyestrone. II. Isolation and Characterization of Urinary Metabolites of 3-Desoxyestrone in Rabbit[1,2]

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The metabolic fate of 3-desoxyestrone, which is used as a lipid-shifting drug, has been investigated with rabbit. Five phenolic and three neutral metabolites were separated from the urine after oral administration of 3-desoxyestrone. The structures of these metabolites were characterized by direct comparison with the authentic samples, respectively (see Chart 1). The biochemical significance of the transformations observed has been discussed.

3-Desoxyestrone (IX) is now widely used for clinical states associated with hypercholesterolemia as a lipid-shifting drug.[4] It was of interest to us to assay the biotransformation products for hypocholesteremic and related physiological activities. The metabolic fate of this drug, however, has not yet been clarified. The present paper deals with the characterization of several metabolites isolated from the rabbit urine after administration of 3-desoxyestrone.

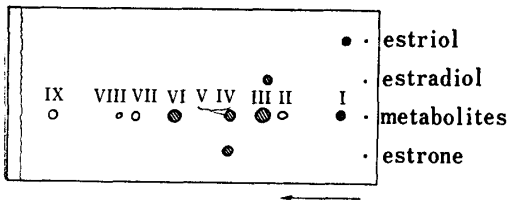

Fig. 1. Thin-Layer Chromatogram of the Metabolites of 3-Desoxyestrone

adsorbent: silica gel G (E. Merck, AG)
developing solvent: hexane–AcOEt (2:1)
staining reagent: conc. H_2SO_4
◍: phenolic ○: neutral

A single dose of a suspension of 3-desoxyestrone was orally given to an adult male rabbit. The urine was collected for the following 48 hours, and hydrolysis of the glucuronides was processed in the usual manner employing beef-liver β-glucuronidase. The hydrolyzate was then extracted with ethyl acetate, and the extract was in turn subjected to solvolysis.[5] Separation of the main metabolites was efficiently achieved by column chromatography on alumina and then, if necessary, by preparative thin-layer chromatography. The metabolites were devided into two groups, the neutral and phenolic, which were distinctly differentiated by staining with Folin-Ciocalteu reagent on the thin-layer chromatogram (see Fig. 1). These metabolites are numerically designated according to the order of decreasing polarity.

Metabolite I could not be crystallized and therefore it was purified by usual acetylation followed by hydrolysis with alkali. This substance showed positive reaction with Folin–Ciocalteu reagent, and its chromatographic behaviors implied the existence of additional two

1) This paper constitutes Part XXIV of the series entitled "Analytical Chemical Studies on Steroids"; Part XXIII: T. Nambara, H. Hosoda, and M. Usui, *Chem. Pharm. Bull.* (Tokyo), **17**, 947 (1969). Preliminary accounts of this work have been presented (*Chem. Pharm. Bull.* (Tokyo), **16**, 383, 1148 (1968)).

2) The following trivial names are used: 3-desoxyestrone, estra-1,3,5(10)-trien-17-one; 3-desoxyestradoil, estra-1,3,5(10)-trien-17β-ol; 3-desoxy-17α-estradiol, estra-1,3,5(10)-trien-17α-ol; estrone, 3-hydroxyestra-1,3,5(10)-trien-17-one; estradiol, estra-1,3,5(10)-triene-3,17β-diol; 17α-estradiol, estra-1,3,5(10)-triene-3,17α-diol; 16,17-epiestriol, estra-1,3,5(10)-triene-3,16β,17α-triol.

3) Location: *Aobayama, Sendai.*

4) A.H. Goldkamp, W.M. Hoehn, R.A. Mikulec, E.F. Nutting, and D.L. Cook, *J. Med. Chem.*, **8**, 409 (1965).

5) S. Burstein and S. Lieberman, *J. Biol. Chem.*, **233**, 331 (1958).

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hydroxyl groups. The chemical shift of C-4-aromatic proton indicated the presence of a hydroxyl group at C–3 rather than at C–2.[6] In addition, the C–17-proton signal appeared as a singlet supporting the 16β,17α-*trans*-structure among four possible 16,17-glycols. In fact, the metabolite proved to be identical with 16,17-epiestriol by mixed melting point and infrared spectra determinations.

The second phenolic metabolite, III, exhibited the coloration characteristic to the estrogens with conc. sulfuric acid and gave the diacetate. Oxidation with Jones reagent[7] provided an oxosteroid, whose chromatographic properties and color reactions were the same as those of estrone. Subsequent reduction of this product with sodium borohydride yielded again a dihydroxy compound, which was, however, obviously distinguishable from III. These results led unequivocally to the assumption that the metabolite would be 17α-estradiol. Indeed, identity of metabolite III with the authentic specimen was justified by usual criteria.

The metabolite IV gave the positive reactions with Zimmermann and Folin–Ciocalteu reagents. This substance was quite similar to estrone but different in respect of the chromatographic properties. Treatment with sodium borohydride furnished a reduction product which proved to be identical with the authentic 2-hydroxy-3-desoxyestradiol.[8] All these evidences together permitted the assignment of the structure 2-hydroxy-3-desoxyestrone to metabolite IV.

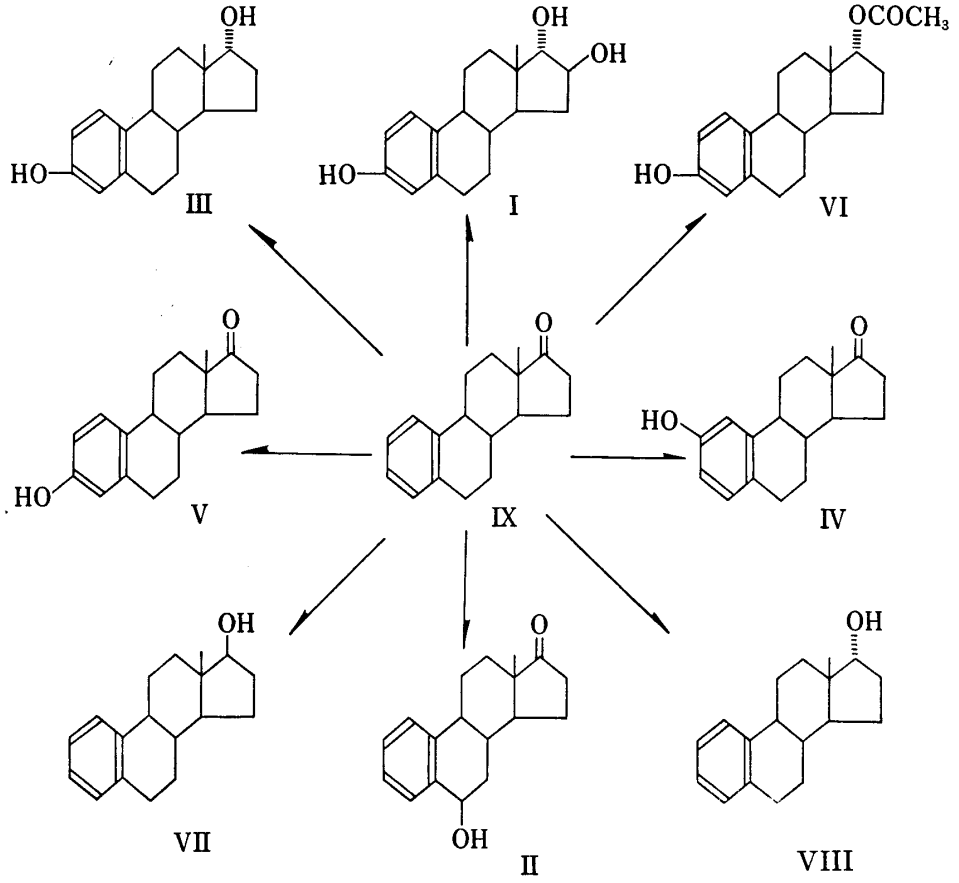

Chart 1

In the mother liquor of metabolite IV the presence of the fourth phenolic metabolite, V, was suggested. Unfortunately this substance could not be separated in pure state because of close similarity in chromatographic behaviors to those of 2-hydroxy-3-desoxyestrone. When

6) J. Fishman and J. Liang, *Tetrahedron*, **24**, 2199 (1968).
7) K. Bowden, I.M. Heilbron, E.R.H. Jones, and B.C.L. Weedon, *J. Chem. Soc.*, **1946**, 39.
8) J. Fishman and M. Tomaz, *J. Org. Chem.*, **27**, 365 (1962).

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converted to the trimethylsilyl derivative, a distinct separation was attained by gas–liquid chromatography on 1.5% SE–30 or 2% OV–17 columns. On the basis of the color reactions and chromatographic constants was drawn a conclusion that the metabolite V should be estrone.

As a remaining phenolic substance metabolite VI was isolated. Both nuclear magnetic resonance and infrared spectra were indicative of the existence of O-acetyl group. The alkaline hydrolysis gave 17α-estradiol, while usual acetylation afforded the diacetate. These results suggested that the metabolite VI would be 17α-estradiol 17-monoacetate. The structure was definitely established by direct comparison with the synthetic sample.[9]

Among the neutral metabolites the most polar one, metabolite II, was isolated as colorless plates. The infrared spectra and Zimmermann reaction were compatible with the presence of the 17-ketone. On the mass spectrum the strong peaks appeared at *m/e* 270 (M^+), 252 (M^+-H_2O) and 141 (benztropylium ion) suggesting a hydroxyl group at C–6.[10] The oxidation product with Jones reagent exhibited the characteristic infrared and ultraviolet absorptions due to the ketone adjacent to the aromatic ring A. In actuality this 6,17-diketone was identical with the authentic sample prepared from 3-desoxyestrone by chromium trioxide oxidation[9]. Based upon these results the structure of this metabolite was assumed to be 6ξ-hydroxy-3-desoxyestrone. Upon comparison with the two epimeric 6-hydroxy-3-desoxyestrones synthesized by the authors,[9] the metabolite II proved to be identical with the 6β-epimer in every respect.

The preparative thin–layer chromatography of the non–polar fraction gave the second neutral metabolite, VII. According to the physical and chemical properties this substance seemed very likely to be 3-desoxyestradiol. Identity of the metabolite with the authentic specimen[11] was rationalized by usual criteria.

In the mother liquor after separation of VII an additional monohydroxy compound, metabolite VIII, was detected on the thin–layer chromatogram. The difficulties were encountered to isolate it in crystalline state because of very small amounts available and of close similarity to VII in physical properties. On trimethylsiliylation, however, metabolite VIII could effectively be separated by gas–liquid chromatography and in consequence, could be characterized as 3-desoxy-17α-estradiol.

Discussions

The occurrence of the phenolic metabolites having hydroxyl group at C–2 or C–3 is of interest in that the existence of aromatic 2- and 3-hydroxylases is indicated. In particular, the isolation of 2-hydroxy-3-desoxyestrone which lacks an oxygen function at C–3, is the first report involving 2-hydroxylation of aromatic ring A, although the catechol estrogens are well known as the novel metabolites.[12] It is to be noted that as for the 3-oxygenated steroids, 17α-estradiol and 16,17-epiestriol were excreted as the main metabolites, whereas only a small amount of estrone was detected. In contrast, no evidence for reduction of 17-oxo group was obtained with the 2-hydroxylated estrogen. These findings imply the possibility that the 17α-hydroxy steroid reductase may require the presence of oxygen function at C–3 or may be sterically hindered by the C–2-hydroxyl group.

The appearance of 17α-estradiol 17-monoacetate is also to be noted. It is well established that the steroidal acetates are sometimes artificially formed during the processing of separa-

9) T. Nambara, M. Numazawa, and H. Takahashi, *Chem. Pharm. Bull.* (Tokyo), in press.
10) H. Budzikiewicz, C. Djerassi, and D.H. Williams, "Structure Elucidation of Natural Products by Mass Spectrometry," Vol II, Holden–Day, Inc., San Francisco, 1964, p. 59.
11) K. Sakakibara, M. Sawai, Y. Suzuki, and K. Chuma, Japan. Patent 4071 (1963) [*C. A.*, **59**, 11607 (1963)].
12) S. Kraychy and T.F. Gallagher, *J. Am. Chem. Soc.*, **79**, 754 (1957); J. Fishman and T.F. Gallagher, *Arch. Biochem. Biophys.*, **77**, 511 (1958).

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tion.[13] The authors examined carefully whether the acetate might be produced in the step of hydrolysis of the urinary conjugates. Nevertheless, it was revealed that O-acetylation under the conditions employed seemed unlikely, and 17α-estradiol was actually metabolized into the acetate *in vivo*. Schubert, *et al.*[14] and YoungLai, *et al.*[15] isolated 3β-acetoxyandrost-5-ene-7,17-dione and 16α-acetoxy-3α-hydroxyandrost-5-en-17-one from the human urine, respectively, which were considered to be the naturally occurring products. Recently, Grosser, *et al.* also reported the acetylation of cortisol.[16] The biochemical introduction of O-acetyl group into the steroids appears to be a novel transformation in the animal kingdom.

The isolation of 16,17-epiestriol is indicative of the presence of the 16β-hydroxylase. Layne and his co-workers reported that so far there could be seen no evidence for urinary metabolites other than 17α-estradiol in rabbit administered with estrone or estradiol.[17] In our experience the excretion of 16,17-epiestriol could also be confirmed in rabbit after administration of estrone.[18] All these phenolic metabolites may presumably be produced through the normal pathway after the initial hydroxylation at C–3.

Next it should be emphasized that hydroxylation did take place at C–6 without disturbance of ring A providing 6β-hydroxy-3-desoxyestrone. It has been demonstrated that both 6β- and 6α-hydroxylases can act on the estrogen substrate to give two 6-hydroxy epimers.[19] In the case of 3-desoxyestrone, however, C–6-hydroxylation occurred exclusively on the β-side of the molecule probably due to lack of the oxygen function at C–3. This polar substance is one of the principal metabolites and hence it is strongly suggested that 6β-hydroxylation may be the compensative bioconversion for common conjugation. In view of the polarization effect the biochemical significance of this transformation must be further investigated.

Two additional 17-hydroxy epimers appeared as the neutral metabolites, of which the 17β-hydroxy compound was found dominant. This result is also different from the case of 3-oxygenated steroids in that the 17α-hydroxy derivative is solely excreted by rabbit.

It is of particular interest that 3-desoxyestrone showed a somewhat different pattern from the normal steroids in the metabolic fate. Further studies on metabolism of 3-desoxysteroids are being extended to C_{19}-series in this laboratory, and the result will be reported in near future. It is also hoped that the quantitative examination of the metabolites together with the conjugates will provide the more precise knowledge on the biotransformation of 3-desoxyestrone.

Experimental

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

Administration of 3-Desoxyestrone and Collection of Urine——In a typical run a single dose of a suspension of 3-desoxyestrone (350 mg) in Tween 80 was injected into a stomach through a catheter, and the urine was collected in bottle containing a few drops of toluene for 48 hr after administration.

Hydrolysis with β-Glucuronidase and Solvolysis——The pooled urine was adjusted to pH 5 with dil. H_2SO_4 and then to pH 4.5 with 0.1 M acetate buffer (10 ml/100 ml of urine) and incubated with beef-liver β-glucuronidase (Tokyo Zoki Kagaku Co.) (300 Fishman units/ml) at 38° for 5 days. The urine was then brought to pH 1 with 50% H_2SO_4, saturated with NaCl (20g/100 ml) and extracted with AcOEt (2×1 vol.). The organic phases were combined and allowed to stand at 38° for 24 hr. The extract was

13) S. Lieberman, K. Dobriner, B.R. Hill, L.F. Fieser, and C.P. Rhods, *J. Biol. Chem.*, **172**, 263 (1948).
14) K. Schubert and K. Wehrberger, *Endokrinologie*, **47**, 290 (1965).
15) E. YoungLai and S. Solomon, *Biochemistry*, **6**, 2040 (1967).
16) B.I. Grosser and L.R. Axelrod, *Steroids*, **9**, 229 (1967); *idem*, *ibid.*, **11**, 827 (1968).
17) D.C. Collins, K.I.H. Williams, and D.S. Layne, *Arch. Biochem. Biophys.*, **121**, 609 (1967).
18) T. Nambara and M. Numazawa, unpublished data.
19) J. Breuer, F. Breuer, H. Breuer, and R. Knuppen, *Z. Physiol. Chem.*, **346**, 279 (1966), and references quoted therein.

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washed with 5% $NaHCO_3$, H_2O and dried over anhydrous Na_2SO_4. After evaporation of solvent a gummy substance was obtained.

Separation of the Metabolites——The gummy extract was submitted to column chromatography on Al_2O_3 (5 g) for removal of the polar resinous substances. After elution with benzene–ether (1:1), metabolite I (20 mg) was eluted with acetone. Eluate obtained with benzene–ether (*ca.* 400 mg) was rechromatographed on Al_2O_3 (15 g), and each 20 ml of effluent was fractionally collected as follows:

Fraction No.	Solvent system	Yield (mg)	Metabolite
1—4	hexane–benzene (3:1)	5	IX
5—8	benzene	5	VII, VIII
9—12	benzene	15	II
13—25	benzene–ether (4:1)	45	III, IV, V, VI
26—27	ether	trace	
28—30	acetone	5	I

Separation of VII and VIII, V and VI was achieved by the preparative TLC using silica gel H (E. Merck, AG) upon repeated developments.

Gas-Liquid Chromatography (GLC)——The apparatus used was Shimadzu Gas Chromatograph Model GC–1C equipped with hydrogen flame ionization detector and stainless steel tube (1.875 m × 3 mm *i.d.*) packed with 1.5% SE–30 on Chromosorb W (60—80 mesh) (A) or 2% OV–17 on Shimalite W (60—80 mesh) (B). The temperatures of column and injection chamber were kept at 215° and 250° (condition GC I) or 230° and 240° (condition GC II), respectively. N_2 was used as a carrier gas at flow rate of 50, 35 and 60 ml/min for conditions GC–IA, –IIA and–IIB. Retention times relative to cholestane (GC–IA 17.4, –IIA 16.0 and –IIB 7.5 min), t_R, are given.

Thin-Layer Chromatography (TLC)——TLC on silica gel G (E. Merck, AG) was carried out by the following systems: TL–I=benzene–ether (4:1); TL–II=hexane–ethyl acetate (2:1); TL–III=benzene–ether (1:1); TL–IV=hexane–ethyl acetate (3:2), and *Rf* values are given.

Identification of the Metabolites[20]

16,17-Epiestriol (Metabolite I)——Elution with acetone and subsequent purification by preparative TLC gave an oily substance (8 mg), which showed positive reaction with Folin–Ciocalteu reagent and yellowish pink staniing with conc. H_2SO_4. Usual acetylation with pyridine and Ac_2O furnished the triacetate as an oily product. NMR ($CDCl_3$ solution) δ: 0.94 (3H, s, 18–CH_3), 2.08, 2.02 (3H, s, 16β–, 17α–$OCOCH_3$), 2.23 (3H, s, 3–$OCOCH_3$), 4.83 (1H, s, 17β–H), 6.76 (1H, s, 4–H). Subsequent hydrolysis with 2.5% methanolic KOH and recrystallization of the hydrolyzate from MeOH–benzene gave metabolite I (3 mg) as colorless prisms. mp 248—250°. Mixed mp on admixture with the authentic sample and infrared spectra comparison showed identity of two samples.

6β-Hydroxy-3-desoxyestrone (Metabolite II)——Elution with benzene and recrystallization of the eluate from aq. MeOH gave metabolite II (8 mg) as colorless plates. mp 216—218°, 225—228° (polymorphism). The metabolite showed negative reaction with Folin–Ciocalteu reagent. IR ν_{max}^{KBr} cm^{-1}: 1725 (5-membered ring C=O), 1404 (active CH_2). NMR ($CDCl_3$ solution) δ: 0.93 (3H, s, 18–CH_3), 4.85 (1H, d, 6α–H). Mass spectrum *m/e*: 270 (M^+), 252 ($M^+ - H_2O$), 141 (benztropilium ion). TLC: TL–II 0.26, –III 0.49, –IV 0.46. GLC (TMS derivative): GC–IIA 0.31, –IIB 0.54. *Anal.* Calcd. for $C_{18}H_{22}O_2$: C, 79.96; H, 8.20. Found: C, 80.25; H, 8.06. The metabolite proved to be entirely identical with the synthetic sample by mixed mp measurement, IR spectra and chromatographic comparisons. Oxidation with Jones reagent and recrystallization of the product from ether gave the 6,17-diketone. mp 180—182°. IR ν_{max}^{KBr} cm^{-1}: 1730 (5-membered ring C=O), 1680 (conjugated C=O). UV λ_{max}^{MeOH} mμ(ε): 252 (17,000), 294 (2200). This diketone was found to be identical with the synthetic sample as judged by mp and IR spectra determinations. Reduction of the metabolite with $NaBH_4$ in MeOH at 0° gave 6β-hydroxy-3-desoxyestradiol. mp 111—114°/155—157°. Mixed mp on admixture with the authentic sample showed no depression.

17α-Estradiol (Metabolite III)——Elution with benzene–ether (4:1) and recrystallization of the eluate from aq. MeOH gave metabolite III (20 mg) as colorless needles. mp 221—222°. The metabolite showed positive reaction with Folin–Ciocalteu reagent and yellowish pink staining with conc. H_2SO_4. IR ν_{max}^{KBr} cm^{-1}: 3360 (OH). TLC: TL–II 0.26, –III 0.57, –IV 0.38. GLC: GC–IA 0.58. Usual acetylation with pyridine and Ac_2O and recrystallization of the product from ether gave the diacetate as colorless needles. mp 140—141°. NMR ($CDCl_3$ solution) δ: 0.76 (3H, s, 18–CH_3), 2.03 (3H, s, 17α–$OCOCH_3$), 2.23 (3H, s, 3–$OCOCH_3$), 4.84 (1H, d, J=6 cps, 17β–H), 6.74 (1H, s, 4–H). Mixed mp on admixture with the authentic sample and IR spectra comparsion showed identity of two samples. Oxidation of the metabolite with Jones reagent furnished estrone (TLC: TL–II 0.34, –III 0.73, –IV 0.51. GLC: GC–IA 0.51), which in turn was led to

20) Melting points were taken on a micro hot stage apparatus and are uncorrected. Infrared spectra measurements were run on Hitachi Model EPI–2 spectrophotometer. Nuclear magnetic resonance spectra were measured on Hitachi Model H–60 spectrometer at 60 Mc in $CDCl_3$; the chemical shifts are quoted as ppm downfield from tetramethylsilane used as an internal standard.

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estradiol (TLC: TL–II 0.24, –III 0.52, –IV 0.36. GLC: GC–IA 0.64) by $NaBH_4$ reduction in MeOH at 0°. Both derivatives showed identical *Rf* and t_R values as the authentic samples, respectively. From these results metabolite III was fully characterized as 17α-estradiol.

2-Hydroxy-3-desoxyestrone (Metabolite IV)——Elution with benzene–ether (4:1) and recrystallization of the eluate from ether gave metabolite IV (4 mg) as colorless needles. mp 201—203°. This substance gave positive reactions with Folin-Ciocalteu and Zimmermann reagents and reddish violet staining with conc. H_2SO_4. IR ν_{max}^{KBr} cm^{-1}: 3420 (OH), 1728 (5-membered ring C=O). Comparison of the metabolite with the authentic sample showed identity in chromatographic constants. TLC: TL–II 0.38, –III 0.76, –IV 0.52. GLC: GC–IA 0.52. Reduction with $NaBH_4$ in MeOH at 0° and recrystallization of the product from acetone–hexane gave 2-hydroxy-3-desoxyestradiol. mp 217—221°. TLC: TL–II 0.18, –III 0.44, –IV 0.31. GLC: GC–IA 0.60. This reduction product was identical with the authentic sample in every respect. Subsequent oxidation with Jones reagent gave again IV.

Estrone (Metabolite V)——The mother liquor of metabolite IV was submitted to the preparative TLC on silica gel H. Elution of the adsorbent corresponding to the spot with $CHCl_3$ gave metabolite V as an oily substance. TLC: TL–II 0.34, –III 0.73, –IV 0.51. GLC: GC–IA 0.51. According to the procedure of Sweeley, *et al.*[21] the metabolite was led to TMS derivative (GLC: GC–IIA 0.56, –IIB 1.02), which showed distinct separation from TMS derivative of metabolite IV (GLC: GC–IIA 0.50, –IIB 0.90).

17α-Estradiol 17-Acetate (Metabolite VI)——Elution with benzene–ether (4:1) and recrystallization of the eluate from aq. MeOH gave metabolite VI (8 mg) as colorless needles. mp 183—184.5°. The metabolite showed positive reaction with Folin–Ciocalteu reagent and yellowish pink staining with conc. H_2SO_4. IR ν_{max}^{KBr} cm^{-1}: 1705 (C=O), 1214 (C–O). NMR ($CDCl_3$ solution) δ: 2.03 (3H, s, 17α-$OCOCH_3$), 4.85 (1H, d, $J=6$ cps, 17β-H), 6.55 (1H, s, 4-H). Mixed mp on admixture with the synthetic sample and IR spectra comparison showed identity of two samples. Usual acetylation with pyridine and Ac_2O gave the 3,17-diacetate, while hydrolysis with 2.5% methanolic KOH gave 17α-estradiol. The structures of these derivatives were justified by TLC employing the authentic specimens.

3-Desoxyestradiol (Metabolite VII)——Elution with benzene and recrystallization of the eluate from MeOH gave metabolite VII (3 mg) as colorless nedeles. mp 116—117°. Mixed melting point on admixture with the authentic sample showed no depression. TLC: TL–I 0.52, –II 0.64, –III 0.71. GLC (TMS derivative): GC–IIA 0.30, –IIB 0.34. Comparison of the metabolite with the authentic sample showed identity in respects with the chromatographic constants. Usual acetylation with pyridine and Ac_2O and oxidation with Jones reagent gave the 17β-acetate and 3-desoxyestrone, respectively. These derivatives were identical with the authentic samples.

3-Desoxy-17α-estradiol (Metabolite VIII)——Elution with benzene gave a trace of metabolite VIII as an oily substance. The metabolite showed negative reaction with Folin–Ciocalteu reagent. TLC: TL–I 0.62, –II 0.70, –III 0.79. GLC (TMS derivative): GC–IIA 0.26, –IIB 0.29. The metabolite was identical with 3-desoxy-17α-estradiol but distinctly different from 3-desoxyestradiol according to TLC and GLC. Oxidation with Jones reagent gave 3-desoxyestrone, identical with the authentic sample in all respects.

3-Desoxyestrone (Administered Steroid)——Elution with hexane–benzene (3:1) and recrystallization of the eluate from ether gave metabolite IX (6 mg) as colorless plates. mp 141—143°. TLC: TL–II 0.89. Mixed mp on admixture with the authentic sample showed no depression.

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21) C.C. Sweeley, R. Bentley, M. Makita, and W.W. Wells, *J. Am. Chem. Soc.*, **85**, 2497 (1963).

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