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Studies on Metabolism of 3-Deoxysteroids. VI. Isolation and Characterization of Urinary Metabolites of 3-Deoxydehydroepiandrosterone in Rabbit [1]

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The metabolic fate of 3-deoxydehydroepiandrosterone (DDHA) in rabbit has been investigated. After oral administration of the steroid, six main metabolites (A–F) were separated from the collected urine specimen. The structures of these biotransformation products could be characterized either by direct comparison with the authentic sample or by leading to the known compound, respectively. The biochemical significance of *in vivo* transformation of C_{19} steroid lacking an oxygen function at C-3 has been discussed.

As a part of studies on metabolism of the modified steroids, the biotransformation of 3-deoxyestrone in rabbit was previously reported.[3] Further interest in 3-deoxysteroids prompted us to explore the metabolic fate of C_{19} steroid lacking an oxygen function at C-3. In this paper we report the isolation and characterization of the urinary metabolites of 3-deoxydehydroepiandrosterone (DDHA) (androst-5-en-17-one) administered to a rabbit.

In a typical run a suspension of the steroid (400 mg/day) in Tween 80 was orally given to an adult female rabbit for two days. The urine was collected for the following 3 days and hydrolysis of the glucuronides was processed in the usual manner employing beef-liver β-glucuronidase. The hydrolyzate was extracted with ether and the aq. layer was then subjected to solvolysis.[4] The result of thin-layer chromatography (TLC) indicated that the nonconjugated steroid fraction thus obtained consisted of several metabolites[5] (see Fig. 1). Separation of these metabolites was efficiently achieved by gradient elution chromatography on alumina using benzene–4%ethanol in benzene as eluent.

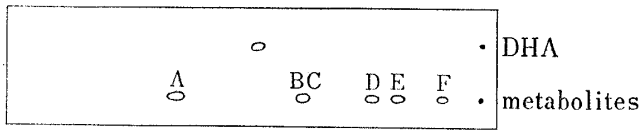

Fig. 1. Thin-Layer Chromatogram of the Metabolites of 3-Deoxydehydroepiandrosterone

adsorbent: silica gel G (E. Merck AG)
developing solvent: benzene–ether (1:1)
staining reagent: conc. H_2SO_4

The most nonpolar metabolite, A, whose chromatographic behaviors were entirely identical with those of androst-5-en-17β-ol, was isolated first. The oxidation of metabolite A with chromium trioxide gave DDHA, which was in turn reduced to the 17β-hydroxyl derivative by metal hydride.

Recrystallization of the second eluate from ethyl acetate gave metabolite C as colorless needles. Chromium trioxide oxidation furnished the diketone which proved to be identical with the authentic 5α-androstane-3,17-dione. Of the four epimeric 5α-androstane-3,17-diols, metabolite C was found to be identical with the 3β,17α-dihydroxylic compound in every respect.

1) This paper constitutes Part XLI of the series entitled "Analytical Chemical Studies on Steroids"; Part XL: T. Nambara and Y.H. Bae, *Chem. Pharm. Bull.* (Tokyo), **18**, 2119 (1970).
2) Location: *Aobayama, Sendai.*
3) *a*) T. Nambara and M. Numazawa, *Chem. Pharm. Bull.* (Tokyo), **16**, 383 (1968); *b*) T. Nambara, M. Numazawa, and H. Takahashi, *ibid.*, **16**, 1148 (1968); *c*) T. Nambara and M. Numazawa, *ibid.*, **17**, 1725 (1969).
4) S. Burstein and S. Liebermann, *J. Biol. Chem.*, **233**, 331 (1958).
5) The metabolites are alphabetically designated according to the order of increasing polarity.

In the mother liquor of metabolite C the presence of a trace amount of metabolite B was suggested according to TLC. Difficulties were encountered to isolate it in pure state because of very small amount available and of close similarity in the physical properties to metabolite C. Upon trifluoroacetylation, however, gas chromatographic separation could be achieved with success on 5% XE-60 column. Thus the structure of metabolite B was identified as androst-5-ene-3β,17α-diol.

Further elution and recrystallization of the eluate from aq. methanol gave metabolite D as colorless needles. Oxidation with Jones reagent[6] gave 5β-androstane-3,17-dione, which on treatment with lithium aluminum hydride was reduced again to the starting compound. These results led to the conclusion that metabolite D should be 5β-androstane-3α,17β-diol. In actuality the identity of the metabolite and authentic sample was evidently demonstrated by the usual criteria.

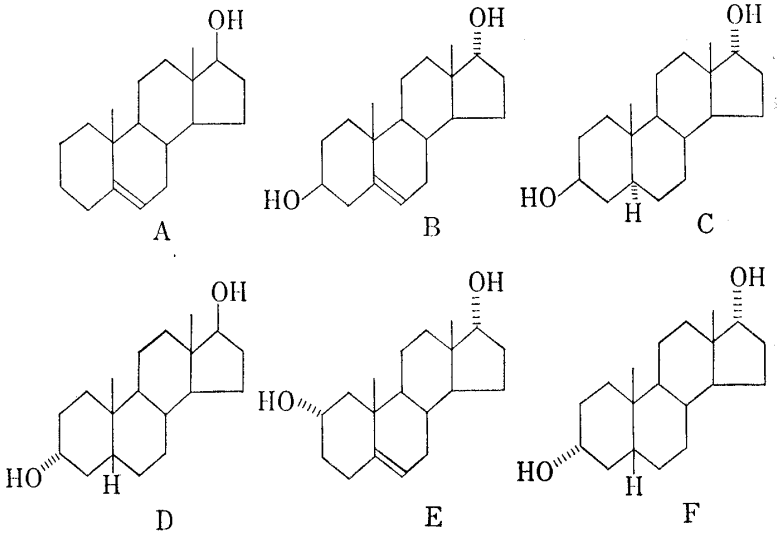

Chart 1

Upon purification of the more polar fraction metabolite E was isolated as colorless prisms. The chromatographic behaviors and mass spectra indicated that this substance would be also a dihydroxyl derivative. The nuclear magnetic resonance spectrum was indicative of the retention of a double bond at C-5 to C-6. Oxidation with Jones reagent[6] furnished the diketone, whose spectroscopic properties did not permit to put the oxygen function at C-3, C-4, or C-7. Based upon these results we assumed that the diketone would be androst-5-ene-2,17-dione. In fact the oxidation product proved to be identical with the authentic sample prepared through an unequivocal route.[7] Configuration of both hydroxyl groups at C-2 and C-17 was tentatively assigned to α rather than β, since there could be seen no remarkable pyridine-induced solvent shift of angular methyl protons in nuclear magentic resonance spectra.[8] On the basis of these evidences the structure of androst-5-ene-2α,17α-diol could be assigned to metabolite E.

The most polar metabolite, F, was separated in the crystalline state by preparative TLC and subsequent recrystallization. The nuclear magnetic resonance and mass spectra revealed the presence of two secondary hydroxyl groups in the saturated androstane nucleus..

6) K. Bowden, I.M. Heilbron, E.R.H. Jones, and B.C.L. Weedon, *J. Chem. Soc.*, **1946**, 39.
7) T. Nambara, H. Takahashi, and Y. Kamikawa, *Chem. Pharm. Bull.* (Tokyo), **18**, 1737 (1970).
8) P.V. Demarco, E. Farkas, D. Doddrell, B.L. Mylari, and E. Wenkert, *J. Am. Chem. Soc.*, **90**, 5480 (1968); T. Nambara, H. Hosoda, and M. Usui, *Chem. Pharm. Bull.* (Tokyo), **17**, 1687 (1969).

Treatment with Jones reagent[6] gave a diketone, whose functional groups would be located in both ring D and six-membered ring as judged by infrared spectra. Direct comparison of this diketone with authentic 5β-androstan-3,17-dione showed the identity of two samples unequivocally. Configuration of both hydroxyl functions at C-3 and C-17 was deduced to be α on the basis of the nuclear magnetic resonance spectra. The chemical shifts of the C-18 and C-19 protons in this metabolite (0.65, 0.94 ppm) were in good accord with the expected values[9] of the 3α,17α-diol (0.66, 0.94 ppm) but not with the 3β,17β-(0.73, 0.98 ppm) and 3β,17α-(0.66, 0.99 ppm) epimers. These evidences together led to the conclusion that metabolite F should be 5β-androstane-3α,17α-diol.

It is of particular interest that hydroxylation occurred at C-2 and C-3 indicating the existence of the corresponding hydroxylase in rabbit. To the best of our knowledge this is the first reported *in vivo* hydroxylation at C-3 in C_{19} steroid, although microbial hydroxylation has been observed with androst-5-en-7-ones.[10] All the 3-oxygenated metabolites would be produced by the enzyme systems involving 3-dehydrogenase, $\Delta^5 \rightarrow \Delta^4$ isomerase and Δ^4-hydrogenase after the initial hydroxylation at C-3.[11] It is also to be noted that DDHA underwent hydroxylation at C-2 without accompanying any disturbance at C-3 and Δ^5-double bond. This result implies that the presence of an oxygen function at C-3 is not necessarily prerequisite for C-2 hydroxylation. As for reduction of the 17-oxo group the administered steroid was principally metabolized into the 17α-hydroxylic compounds with exception of 3-deoxy- and 5β-steroids. This metabolic pattern appears to be similar to that of 3-deoxyestrone.[3c]

So far as we know it seems very likely that 3-oxygenated compounds are the major metabolites formed from 3-deoxysteroids, and C-3 hydroxylase is widely distributed in the animal kingdom. The present results together with the previous findings strongly suggest that 3-deoxysteroids, designed to minimize the undesirable side effects by removal of an oxygen function at C-3, are principally metabolized along the normal pathway after the hydroxylation at C-3.

Experimental[12]

Material——3-Deoxydehydroepiandrosterone (DDHA) (androst-5-en-17-one) was prepared from androst-5-en-17β-ol[13] by Jones oxidation.[6] mp 108—109.5°. $[\alpha]_D^{23}$ −24.0° (c=0.73, $CHCl_3$). *Anal.* Calcd. for $C_{19}H_{28}O$: C, 83.77; H, 10.36. Found: C, 83.97; H, 10.43.

Animal——An adult female rabbit weighing about 2.5 kg was housed in a cage that was designed to minimize fecal contamination of the urine.

Administration of DDHA and Collection of Urine——In a typical run a single dose of suspension of DDHA (400 mg/day) in Tween 80 was orally given for two days, and the urine was collected in a bottle containing a few drops of toluene for 3 days after administration.

Hydrolysis with β-Glucuronidase and Solvolysis——The pooled urine was adjusted to pH 5 with 50% H_2SO_4 and then to pH 4.7 with 0.1M acetate buffer (10 ml/100 ml of urine) and incubated with beef-liver β-glucuronidase (Tokyo Zōkikagaku Co.) (300 Fishman U/ml) at 37° for 5 days. The urine was extracted with ether and then aq. layer was brought to 2N H_2SO_4 solution with 50% H_2SO_4, saturated with NaCl

9) N.S. Bhacca and D.H. Williams, "Application of NMR Spectroscopy in Organic Chemistry," Holden-Day, Inc., 1964, pp. 14—32; R.F. Zürcher, *Helv. Chim. Acta*, **44**, 1380 (1961); *idem*, *ibid.*, **46**, 2054 (1963).

10) P.C. Cherry, E.R.H. Jones, and G.D. Meakins, *Chem. Commun.*, **1966**, 587.

11) In a separate experiment the urinary excretion of the same 3-oxygenated metabolites (B, D and F) could be confirmed after oral administration of dehydroepiandrosterone (DHA) in rabbit, *a*) T. Nambara, H. Takahashi, and Y. Kamikawa, unpublished data.

12) All melting points were taken on a micro hot-stage apparatus and are uncorrected. Infrared (IR) spectra measurements were run on Hitachi Model EPI-2 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were obtained on Hitachi Model H-60 spectrometer at 60 Mc; the chemical shifts are quoted as ppm downfield from tetramethylsilane used as an internal standard. Mass (MS) spectra were measured by Hitachi Model RMU-7 spectrometer.

13) R.E. Marker, E.L. Wittle, and B.F. Tullar, *J. Am. Chem. Soc.*, **62**, 223 (1940).

(20 g/100 ml) and extracted with AcOEt. The organic phases were combined and allowed to stand at 37° for 24 hr. The extract was combined with the glucuronide fraction previously separated and washed with 5% $NaHCO_3$, H_2O and dried over anhydrous Na_2SO_4. Evaporation of solvent *in vacuo* gave a gummy substance.

Separation of Metabolites——The gummy residue was submitted to gradient elution column chromatography on Al_2O_3 (activity II, 40 g) with use of benzene (500 ml)–4% EtOH in benzene (500 ml) as eluent and fractions of 10 ml were collected as follows:

Fraction No.	Metabolite	Weight (mg)
4—6	A	2
24—25	B,C	18
27—30	D	3
31—35	E	31
35—38	E,F	5

Separation of E and F 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 a hydrogen flame ionization detector and stainless steel tube (3.375 m×3 mm i.d.) packed with 1.5% SE-30 on Chromosorb W (60—80 mesh) (A), 2% OV-17 on Shimalite W (60—80 mesh) (B), 5% XE-60 on Chromosorb W (60—80 mesh) (C) or 1.5% QF-1 on Chromosorb W (60—80 mesh) (D). The column, injection chamber, and detector were maintained at 230°, 250° and 250° (condition GC-I), 190°, 250° and 240° (condition GC-II) or 180°, 200° and 210° (condition GC-III), respectively. N_2 was used as a carrier gas at flow rate of 50, 60 and 60 ml/min for conditions GC-I, -II and -III, respectively. Retention times relative to cholestane (GC-IA 3.6, -IB 7.0, -IIC 38.0, -IID 7.2 and -IIIC 54.6 min) were given.

Thin-Layer Chromatography (TLC)——TLC on silica gel G (E. Merck AG) was carried out by the following systems: TL-I=benzene–ether (1:1); TL-II=hexane–AcOEt (7:3).

Identification of the Metabolites Androst-5-en-17β-ol (Metabolite A)——Gradient elution chromatography gave metabolite A (2 mg), which showed violet staining with conc. H_2SO_4 on thin-layer plate. TLC: TL-I 0.63. GLC: GC-IA 0.36, -IB 0.28. Oxidation with Jones reagent[6] gave androst-5-en-17-one, which on reduction with $NaBH_4$ in MeOH was transformed again to androst-5-en-17β-ol.

Androst-5-ene-3β,17α-diol (Metabolite B)——From the mother liquor of metabolite C a trace amount of metabolite B was separated. This substance showed purple staining with conc. H_2SO_4 on thin-layer plate. TLC: TL-I 0.29. GLC (TMS derivative): GC-IIC 0.46, -IID 0.50; (TFA derivative): -IIIC 0.68.

5α-Androstane-3β,17α-diol (Metabolite C)——Recrystallization from AcOEt gave metabolite C (18 mg) as colorless needles. mp 212—214°. This substance showed yellowish brown coloration with conc. H_2SO_4 on thin-layer plate. TLC: TL-I 0.29. NMR ($CDCl_3$ solution) δ: 0.65 (3H, s, 18-CH_3), 0.80 (3H, s, 19-CH_3), 3.65 (2H, m, >C<u>H</u>OH). GLC (TMS derivative): GC-IIC 0.46, -IID 0.50; (TFA derivative): GC-IIIC 0.73. Oxidation with Jones reagent[6] gave the diketone (TLC: TL-II 0.47) which was identical with 5α-androstane-3,17-dione in every respect. The metabolite proved to be 5α-androstane-3β,17α-diol among four possible 3,17-dihydroxylic epimers by mixed melting point measurement and IR spectra comparison.

5β-Androstane-3α,17β-diol (Metabolite D)——Recrystallization from aq. MeOH gave metabolite D (3 mg) as colorless needles. mp 224—230°. This substance showed purple coloration with conc. H_2SO_4 on thin-layer plate. TLC: TL-I 0.20. Oxidation with Jones reagent[6] gave 5β-androstane-3,17-dione (TLC: TL-II 0.42), which on reduction with $LiAlH_4$ was backed to 5β-androstane-3α,17β-diol. Mixed melting point on admixture with the authentic sample showed no depression and IR spectra of two samples were identical in all respects.

Androst-5-ene-2α,17α-diol (Metabolite E)——Recrystallization from AcOEt gave metabolite E (33 mg) as colorless prisms. mp 205—208°. This substance showed violet staining with conc. H_2SO_4 on thin-layer plate. TLC: TL-I 0.14. MS *m/e*: 290 (M^+), 272 (M^+-H_2O), 254 (M^+-$2H_2O$). NMR ($CDCl_3$ solution) δ: 0.67 (3H, s, 18-CH_3), 1.00 (3H, s, 19-CH_3), 3.72 (2H, m, >C<u>H</u>OH), 5.35 (1H, m, C_6–H); (pyridine solution) δ: 0.74 (3H, s, 18-CH_3), 1.12 (3H, s, 19-CH_3). Oxidation with Jones reagent[6] gave androst-5-ene-2,17-dione. mp 139—141°. IR ν_{max}^{KBr} cm^{-1}: 1715 (six-membered ketone), 1735 (five-membered ketone). This diketone proved to be identical with the synthetic specimen[7] as judged by mixed melting point and IR spectra determinations.

5β-Androstane-3α,17α-diol (Metabolite F)——Recrystallization from AcOEt gave metabolite F (3 mg) as colorless needles. mp 232—233°. This substance showed violet staining with conc. H_2SO_4 on thin-layer plate. TLC: TL-I 0.07. NMR ($CDCl_3$ solution) δ: 0.65 (3H, s, 18-CH_3), 0.94 (3H, s, 19-CH_3), 3.65 (2H, m, >C<u>H</u>OH). MS *m/e*: 292 (M^+), 274 (M^+-H_2O), 256 (M^+-$2H_2O$). Oxidation with Jones reagent[6] gave the diketone. mp 121—123°. IR ν_{max}^{KBr} cm^{-1}: 1718 (six-membered ketone), 1743 (five-membered ketone). This diketone proved to be 5β-androstane-3,17-dione by mixed melting point measurement with the authentic sample and IR comparison. The chemical shifts of C-18 and C-19 protons calcu lated for three possible 5β-androstane-3,17-diols by additivity rule were as follows: 3α,17α (0.66, 0.94 ppm), 3β,17β (0.73, 0.98 ppm) and 3β,17α (0.66, 0.99 ppm).

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