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Preparation and Stability in Solution of Androstenedione 3-Enol Glucosiduronate

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4-^{14}C-Androstenedione 3-enol glucosiduronate (^{14}C-AEG) was synthesized and its stability was studied by analyzing liberated steroids. Incubation of ^{14}C-AEG with human urine at 37° for 48 hr resulted in 49% hydrolysis. ^{14}C-AEG was fairly stable in neutral and basic media, but rapidly hydrolyzed at acidic pH. ^{14}C-AEG was solvolyzed almost quantitatively with acidic ethyl acetate in the same way as steroid sulfates. By incubation with β-glucuronidase (Ketodase, *Helix pomatia*, and bacterial β-glucuronidase) at pH 6.2 for 3 hr, 64—71% of ^{14}C-AEG was hydrolyzed. Hydrolysis with *Helix pomatia* β-glucuronidase was inhibited by D-glucaro-(1→4)-lactone. Analyses of liberated steroids by TLC revealed the formation of artifacts (polar steroids) in all cases so far examined. One of them was identified as 6β-hydroxyandrostenedione.

The presence of the steroid enol glucosiduronate in biological materials was first suggested by Schubert,[2] who identified androstenedione[3] as a product of β-glucuronidase hydrolysis of material isolated from urine of individuals injected with testosterone. More convincing evidence for the *in vivo* formation of the enol glucosiduronate was adduced by Wotiz, *et al.*,[4] who identified 11β-hydroxy-17-oxoandrosta-3,5-dien-3-yl-β-D-glucopyranosiduronate in the urine of rats receiving androst-4-ene-3,11,17-trione (adrenosterone). The existence of a 1,3-dioxo-C_{21}-steroid 3-enol glucosiduronate in the urine of a hypertensive newborn child was reported by Edwards, *et al.*[5] Quite recently, Rhamy, *et al.*[6] have reported the isolation and identification of androstenedione 3-enol glucopyranosiduronate from the blood of a woman with an interstitial cell ovarian tumor after the administration of 4-^{14}C-testosterone.

Meanwhile, the synthesis of steroid 3-enol glucosiduronates was reported by Wotiz, *et al.*[7] Using the synthetic androstenedione 3-enol glucosiduronate (AEG), Wakabayashi, *et al.*[8] demonstrated that AEG was hydrolyzed rapidly at acidic pH in contrast to non-enolic glucosiduronic acids and by incubation with β-glucuronidase preparations. *In vitro* metabolism of AEG was subsequently investigated by Wotiz,[9] who incubated AEG with rat-liver mince, suggesting that the conjugate may undergo further metabolic change without obligatory cleavage.

Although indirect evidences bearing on the existence of enol glucosiduronates as *in vivo* metabolites of steroids have been accumulating as cited above, unequivocal identification of the conjugate is required before any definite conclusion concerning this problem is drawn. In order to study the properties of the enol glucosiduronate in more detail, synthesis of 4-^{14}C-

1) Location: *a) Shibakoen-1-chome, Minato-ku, Tokyo*; *b) Takada-3-chome, Toshima-ku, Tokyo.*

2) K. Schubert, *Acta Endocrinol.*, **27**, 36 (1958).

3) Following trivial names are used: androstenedione, androst-4-ene-3,17-dione; testosterone, 17β-hydroxy-androst-4-en-3-one; androstenedione 3-enol glucosiduronate, 17-oxoandrosta-3,5-dien-3-yl-β-D-glucopyranosiduronate; 6β-hydroxyandrostenedione, 6β-hydroxyandrost-4-ene-3,17-dione; 6-oxoandrostenedione, androst-4-ene-3,6,17-trione; androsterone, 3α-hydroxy-5α-androstan-17-one.

4) H.H. Wotiz and W.H. Fishman, *Steroids*, **1**, 211 (1963).
5) R.W.H. Edwards and D.J.H. Trafford, *Biochem. J.*, **108**, 185 (1968).
6) K. Rhamy and H.E. Hadd, *Steroids*, **22**, 719 (1973).
7) *a)* H.H. Wotiz, E. Smakula, N.N. Lichtin, and J.H. Leftin, *J. Am. Chem. Soc.*, **81**, 1704 (1959); *b)* E. Smakula, J.H. Leftin, and H.H. Wotiz, *ibid.*, **81**, 1708 (1959).
8) M. Wakabayashi, H.H. Wotiz, and W.H. Fishman, *Biochim. Biophys. Acta*, **48**, 198 (1961).
9) H.H. Wotiz, *Biochim. Biophys. Acta*, **60**, 28 (1962).

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androstenedione 3-enol glucosiduronate (^{14}C-AEG) as well as of AEG is reported in the present work. Using ^{14}C-AEG, stability and enzymic hydrolysis of AEG were investigated by analyzing liberated steroids.

Material and Method

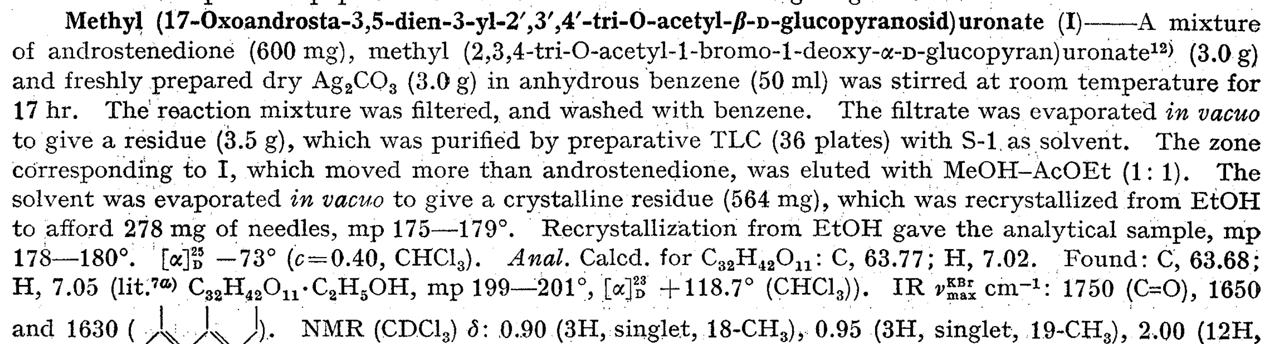
Melting points were determined on a Kofler block and are uncorrected. Infrared (IR) spectra were determined in potassium bromide disks on Hitachi EPI-32 spectrometer. Optical rotations were determined on Hitachi PO-B polarimeter. Nuclear magnetic resonance (NMR) spectra were measured in $CDCl_3$ at 60 MHz, using Hitachi Model R-20A 60 MHz spectrometer. Chemical shifts are expressed in δ (ppm) with tetramethylsilane as internal standard.

Thin-Layer Chromatography (TLC)——TLC was performed on 5×20 cm glass plate coated with 0.5 mm thick film of Silica gel GF (Merck). Preparative TLC was done on 20×20 cm glass plate (1 mm thickness). The reference steroids were applied at the edge of the plate. The following solvent systems were used: S-1, benzene–EtOH (19:1); S-2, $CHCl_3$–MeOH–tetrahydrofuran-28% aqueous ammonia (6:4:1:1); S-3, $CHCl_3$–acetone (29:1); S-4, $CHCl_3$–EtOH (9:1). The plate was developed 2 to 3 times with the same solvent system. The reference steroids with 4-en-3-one or 3,5-diene groupings were detected under a short wave ultraviolet lamp. The radioactivity was detected with an Aloka autoscanner. The radioactive spots were scraped and eluted with appropriate solvents. The reference steroids were visualized by spraying the rest of the plate with H_2SO_4, followed by heating.

Recrystallization——The identity and purity of the steroid were confirmed by adding 13—18 mg of an authentic steroid and recrystallizing the mixture from the appropriate solvent to constant specific activity. The following solvent systems were used for recrystallization: A, MeOH; B, AcOEt–cyclohexane; C, acetone–hexane; D, benzene–petroleum ether.

Measurement of Radioactivity——The radioactivity was measured in Nuclear-Chicago Mark I liquid scintillation spectrometer. The sample aliquot, which consisted of 0.5 or 1.0 ml of MeOH, EtOH, or aqueous solution, was dissolved in 10 ml of the scintillation fluid containing 5 g of PPO or 2,5-diphenyloxazole and 300 mg of 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene per liter of toluene. One ml of BIOSOLV BBS-3 (Beckman) was added to 0.5 ml of the aqueous sample to obtain homogeneous counting fluid. The counting efficiency for each sample was determined by reference to a calibration curve plotted from a set of quenched standards using external ^{133}Ba source and a channel ratio method. Efficiency of ^{14}C counting was about 80%. All the radioactivities are expressed in dpm.

Steroids and Reagents——4-^{14}C-Testosterone (58.8 mCi/mmole) was purchased from New England Nuclear, Boston. 4-^{14}C-Androstenedione was prepared by oxidation of 4-^{14}C-testosterone as follows. ^{14}C-Testosterone (6.00×10^7 dpm) in 2 ml of acetone was treated with 5 drops of Jones reagent (2.7 g of CrO_3 and 2.3 ml of conc. H_2SO_4 diluted with water to 10 ml). The reaction mixture was allowed to stand at room temperature for 15 min, then poured into ice water and extracted with ether. The organic layer was washed with water, dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to afford a residue, which was purified by preparative TLC (2 plates) with solvent system S-1. The zone corresponding to androstenedione was scraped and eluted with AcOEt–MeOH (1:1). The solvent was evaporated *in vacuo* and the resulting 4-^{14}C-androstenedione (5.41×10^7 dpm) was mixed with androstenedione to give a specific activity of 7.58×10^6 dpm/mg. 6β-Hydroxyandrostenedione, 6-oxoandrostenedione, and androst-4-ene-3β,6β,17β-triol were prepared according to the procedure of Amendolla, *et al.*[10] Other reference steroids used in this study were described in the previous paper.[11] All other chemicals were of reagent grade.

Methyl (17-Oxoandrosta-3,5-dien-3-yl-2′,3′,4′-tri-O-acetyl-β-D-glucopyranosid)uronate (I)——A mixture of androstenedione (600 mg), methyl (2,3,4-tri-O-acetyl-1-bromo-1-deoxy-α-D-glucopyran)uronate[12] (3.0 g) and freshly prepared dry Ag_2CO_3 (3.0 g) in anhydrous benzene (50 ml) was stirred at room temperature for 17 hr. The reaction mixture was filtered, and washed with benzene. The filtrate was evaporated *in vacuo* to give a residue (3.5 g), which was purified by preparative TLC (36 plates) with S-1 as solvent. The zone corresponding to I, which moved more than androstenedione, was eluted with MeOH–AcOEt (1:1). The solvent was evaporated *in vacuo* to give a crystalline residue (564 mg), which was recrystallized from EtOH to afford 278 mg of needles, mp 175—179°. Recrystallization from EtOH gave the analytical sample, mp 178—180°. $[\alpha]_D^{25}$ −73° (c=0.40, $CHCl_3$). *Anal.* Calcd. for $C_{32}H_{42}O_{11}$: C, 63.77; H, 7.02. Found: C, 63.68; H, 7.05 (lit.[7a] $C_{32}H_{42}O_{11} \cdot C_2H_5OH$, mp 199—201°, $[\alpha]_D^{23}$ +118.7° ($CHCl_3$)). IR ν_{max}^{KBr} cm^{-1}: 1750 (C=O), 1650 and 1630 (). NMR ($CDCl_3$) δ: 0.90 (3H, singlet, 18-CH_3), 0.95 (3H, singlet, 19-CH_3), 2.00 (12H,

10) C. Amendolla, G. Rosenkranz, and F. Sondheimer, *J. Chem. Soc.*, **1954**, 1226.
11) M. Matsui, F. Abe, K. Kimura, and M. Okada, *Chem. Pharm. Bull.* (Tokyo), **20**, 1913 (1972).
12) G.N. Bollenback, J.W. Long, D.G. Benjamin, and J.A. Lindquist, *J. Am. Chem. Soc.*, **77**, 3310 (1955).

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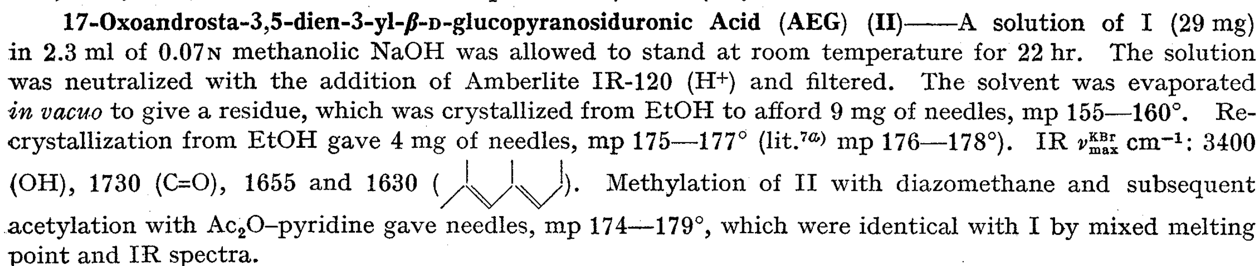
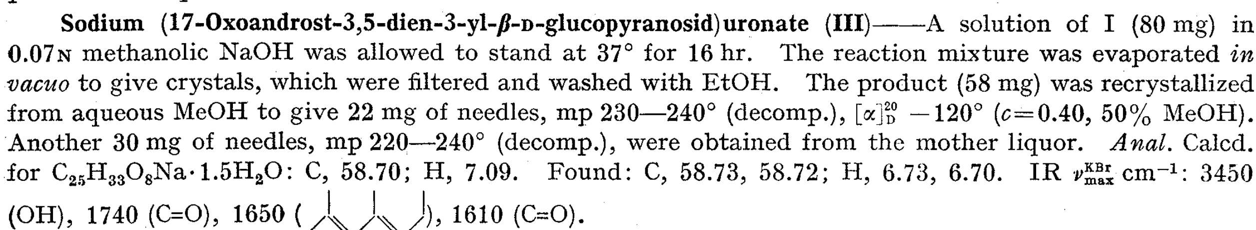
singlet, 4×$OCOCH_3$), 3.75 (3H, singlet, OCH_3), 4.12 (1H, multiplet, H-5′), 4.90—5.40 (6H, multiplet, H-4, H-6, H-1′, H-2′, H-3′ and H-4′). Mass Spectrum *m/e*: 602 (M^+).

17-Oxoandrosta-3,5-dien-3-yl-β-D-glucopyranosiduronic Acid (AEG) (II)——A solution of I (29 mg) in 2.3 ml of 0.07N methanolic NaOH was allowed to stand at room temperature for 22 hr. The solution was neutralized with the addition of Amberlite IR-120 (H^+) and filtered. The solvent was evaporated *in vacuo* to give a residue, which was crystallized from EtOH to afford 9 mg of needles, mp 155—160°. Recrystallization from EtOH gave 4 mg of needles, mp 175—177° (lit.[7a]) mp 176—178°). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1730 (C=O), 1655 and 1630 (). Methylation of II with diazomethane and subsequent acetylation with Ac_2O–pyridine gave needles, mp 174—179°, which were identical with I by mixed melting point and IR spectra.

Sodium (17-Oxoandrost-3,5-dien-3-yl-β-D-glucopyranosid)uronate (III)——A solution of I (80 mg) in 0.07N methanolic NaOH was allowed to stand at 37° for 16 hr. The reaction mixture was evaporated *in vacuo* to give crystals, which were filtered and washed with EtOH. The product (58 mg) was recrystallized from aqueous MeOH to give 22 mg of needles, mp 230—240° (decomp.), $[\alpha]_D^{20}$ −120° (*c*=0.40, 50% MeOH). Another 30 mg of needles, mp 220—240° (decomp.), were obtained from the mother liquor. *Anal.* Calcd. for $C_{25}H_{33}O_8Na \cdot 1.5H_2O$: C, 58.70; H, 7.09. Found: C, 58.73, 58.72; H, 6.73, 6.70. IR ν_{max}^{KBr} cm^{-1}: 3450 (OH), 1740 (C=O), 1650 (), 1610 (C=O).

Methyl (4-^{14}C-17-Oxoandrosta-3,5-dien-3-yl-2′,3′,4′-tri-O-acetyl-β-D-glucopyranosid)uronate (IV)——A mixture of 4-^{14}C-androstenedione (3.79×10^7 dpm, 5.0 mg), methyl (2,3,4-tri-O-acetyl-1-bromo-1-deoxy-α-D-glucopyran)uronate (50 mg) and freshly prepared Ag_2CO_3 (50 mg) in anhydrous benzene (3.0 ml) was treated as described in the preparation of I. The reaction mixture was purified by preparative TLC (1 plate) as described above to afford IV (1.28×10^7 dpm).

4-^{14}C-17-Oxoandrosta-3,5-dien-3-yl-β-D-glucopyranosiduronate (^{14}C-AEG) (V)——A solution of IV (1.27×10^7 dpm) in 0.04N methanolic NaOH (1.0 ml) was kept at 37° for 16 hr. The solution was evaporated *in vacuo* and separated by preparative TLC (1 plate) with solvent system S-2. The zone corresponding to III was scraped, eluted with MeOH, and evaporated *in vacuo* to yield crude V fraction (9.14×10^6 dpm). The crude V fraction (9.05×10^6 dpm) was chromatographed on a column of Celite 545 (40 g) using solvent system, 2,2,4-trimethylpentane–*tert*-BuOH–0.8N NH_4OH (2:5:5).[13] One hold-back volume was 80 ml. Hold-back volumes 1—2 contained the free steroid (1.12×10^6 dpm) and hold-back volumes 5—7 contained the glucosiduronate (7.32×10^6 dpm). The purity of the glucosiduronate fraction was determined by adding 11.32 mg of III and 200 μg of NaOH in MeOH (0.1 ml) to the fraction (7.80×10^4 dpm) and recrystallizing the mixture from MeOH to constant specific activity: 6020 dpm/mg, calculated: 6890 dpm/mg (87% purity). The glucosiduronate fraction (6.25×10^6 dpm) was further chromatographed on Celite 545 as described above to give the purified glucosiduronate fraction (^{14}C-AEG, 4.96×10^6 dpm). The purity was determined in the following way: ^{14}C-AEG fraction (9.08×10^4 dpm) was mixed with 11.84 mg of III and 170 μg of NaOH in MeOH (0.1 ml) and recrystallized from MeOH to constant specific activity: 7510 dpm/mg, calculated: 7670 dpm/mg (98% purity). TLC examination of ^{14}C-AEG with S-2 as solvent revealed the appearance of 96% of the radioactivity in the glucosiduronate fraction. The ^{14}C-AEG (4.41×10^6 dpm, 7.58×10^6 dpm/mg) was dissolved in EtOH (3.0 ml) and kept in a refrigerator.

Distribution of ^{14}C-AEG between Water and Organic Solvent——The EtOH solution (10 μl) of ^{14}C-AEG (1.47×10^4 dpm) was dissolved in 50 ml of water and extracted 3 times with 50 ml of commercial ether, peroxide free ether, methylene dichloride, or AcOEt. Each combined organic layer was washed with 50 ml of water, dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. The residue was dissolved in 10.0 ml of EtOH. The combined aqueous fraction was evaporated *in vacuo* to afford 10.0 ml of aqueous solution. The radioactivities in two fractions were measured. Peroxide free ether was prepared by passing the distilled ether through a column of Aluminium oxide (Merck), just before use.

Stability of ^{14}C-AEG in Human Urine——Ethanol solution (30 μl) of ^{14}C-AEG (4.41×10^4 dpm) or ^{14}C-androstenedione (4.46×10^4 dpm) was added to 50 ml of normal 24-hr human urine (pH 6.3, 960 ml), which was filtered through Millipore filter after centrifugation at 1700×***g*** for 30 min. The urine was kept at 37°. After 48 and 120 hr, 20.0 ml of the urine was taken out and extracted with ether. The ether layer was washed with water, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The residue was dissolved in 5.0 ml of EtOH (liberated steroid fraction). The aqueous layer was evaporated *in vacuo* to give 5.0 ml of aqueous solution (glucosiduronate fraction). Radioactivities of both fractions were counted. Subsequently, the liberated steroids were examined by TLC with S-3 as solvent. Each radioactive peak on TLC was scraped, eluted with MeOH, and concentrated *in vacuo* to give a residue, which was dissolved in 5.0 or 10.0 ml of EtOH and the radioactivity was measured.

Stability of ^{14}C-AEG in Buffer Solutions——^{14}C-AEG (4.41×10^4 dpm) was added to 50 ml of buffer

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13) P.K. Siiteri, *Steroids*, 2, 687 (1963).

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solutions and allowed to stand at 37°. After 1, 2, 3, 4, 5, 6, 24, 48, 72, 120, or 144 hr, an aliquot (5.0 ml) of the solutions was treated as described above. Buffer solutions used were as follows: 0.1M acetate buffer at pH 3.5, 4.5, and 5.2; 0.1M phosphate buffer at pH 6.2, 7.0, and 8.0.

Solvolysis of ^{14}C-AEG——^{14}C-AEG (3.85×10^4 dpm) was added to 50 ml of acidic AcOEt, which was saturated with 2M H_2SO_4 and kept at 37° for 16 hr according to the procedure of Vihko.[14] The AcOEt layer was washed with 5% $NaHCO_3$ and water, dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to give a residue, which was dissolved in 10.0 ml of EtOH (liberated steroid fraction). The aqueous layer was evaporated *in vacuo* to yield 20.0 ml of aqueous solution (glucosiduronate fraction). Radioactivities in both fractions were counted. The liberated steroids were examined by TLC as described above.

Stability of ^{14}C-AEG during the Isolation Procedures[11]——^{14}C-AEG (1.24×10^5 dpm) was added to a mixture of 0.25M sucrose (3.0 ml) and 0.2M Tris buffer (pH 7.2, 3.5 ml). After addition of 50 ml of CH_2Cl_2–MeOH (1 : 4), the mixture was extracted with MeOH (200 ml). After evaporation of the solvent *in vacuo*, the residue was dissolved in water and extracted with ether. The ether layer was treated as above to afford the liberated steroid fraction. The aqueous layer was evaporated under reduced pressure just to eliminate ether and passed through a XAD-2 (100 g) column. The column was washed with 400 ml of water and eluted with 400 ml of MeOH following the procedure of Bradlow.[15] The MeOH fraction was evaporated *in vacuo*, incubated at 37° with 50 ml of acidic AcOEt, and treated as described above.

Hydrolysis of ^{14}C-AEG with β-Glucuronidase Preparations——^{14}C-AEG (4.41×10^4 dpm) was incubated at 37° with 20000 units of Ketodase (beef liver β-glucuronidase) (5000 U/ml, Warner-Chilcott), β-glucuronidase preparation from *Helix pomatia* (100000 U/ml, Advance Kasei), or bacterial type II β-glucuronidase (50000 U/g, Sigma Chem. Co.) in 30 ml of 0.1M phosphate buffer (pH 6.2). After 3, 5, 24, 48, and 72 hr, an aliquot (5.0 ml) of the solution was extracted with ether and treated as described above. Inhibition of the hydrolysis was examined by adding D-glucaro-(1→4)-lactone (20 mg) to the same incubation media described above.

Result and Discussion

Synthesis of 17-Oxoandrosta-3,5-dien-3-yl-β-D-glucopyranosiduronic Acid (AEG) (II)

Methyl (17-oxoandrosta-3,5-dien-3-yl-2′,3′,4′-tri-O-acetyl-β-D-glucopyranosid) uronate (I) was prepared by the Koenigs-Knorr reaction according to the procedure reported earlier.[7a] Satisfactory results were obtained in the elemental analysis and mass spectrum, and the IR spectrum coincided with that of Wotiz, *et al.*[7b] However, the specific rotation (−73°) was entirely different from theirs (+119°).[7a] While, they reported that AEG was levorotatory (−119°).[7a] The value (−73°) is compatible with those of AEG (−119°) and its sodium salt (III) (−120°). The 3,5-diene structure was quite evident from the appearance of two bands at 1650 and 1630 cm^{-1} in the IR spectrum, which were ascribable to the conjugated –C=C– stretching vibrations.[7b] Furthermore, 60 MHz NMR spectrum showed that H-4 and H-6 proton signals were overlapped with H-1′, H-2′, H-3′, and H-4′ proton signals at 4.9—5.4 ppm, indicating the olefinic nature of H-4 and H-6 protons. Low field shift of the anomeric proton (H-1′) signal revealed that the glucuronic acid was conjugated through enol type alcohol, because H-1′ proton signal of the β-D-glucopyranosiduronate, in which glucuronic acid is linked with saturated aliphatic alcohol, appeared at 4.5—4.7 ppm as doublet.[16] Besides optical rotation, the NMR spectrum showed a typical pattern of the β-D-glucopyranosiduronate[16]: H-2′, H-3′, and H-4′ proton signals appeared in the narrow range (0.5 ppm) and H-5′ proton signal was a deformed multiplet, due to virtual long range coupling of H-5′ proton with H-3′ proton. Subsequently, II was prepared by hydrolysis of I. Acetylation followed by methylation of II yielded I, thus confirming the structure of II. II was not a stable compound and recrystallization of II always resulted in the formation of appreciable amount of androstenedione. However, its sodium salt (III) was more stable than II.

Employing the similar procedures, ^{14}C-AEG was prepared from ^{14}C-androstenedione. ^{14}C-AEG was not stable on silica gel plate and was partly hydrolyzed to give ^{14}C-androstene-

14) R. Vihko, *Acta Endocrinol., Suppl.*, No. 109 (1966).
15) H.L. Bradlow, *Steroids*, **11**, 265 (1968).
16) M. Matsui and M. Okada, *Chem. Pharm. Bull.* (Tokyo), **18**, 2129 (1970); *idem, ibid.*, **20**, 1033 (1972).

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dione. Therefore, the purification of ^{14}C-AEG was finally performed by Celite column chromatography. The purity of ^{14}C-AEG was 98%, determined by reverse isotope dilution method.

Distribution of ^{14}C-AEG between Water and Organic Solvent

^{14}C-AEG was partitioned between several organic solvents and water. Extraction with ethyl acetate or peroxide-free ether (freshly prepared) showed that 97% of the radioactivity remained in the aqueous layer. While, 10 and 14% of the radioactivity appeared in the organic layer in the case of methylene dichloride and commercial ether respectively. Thus, peroxide-free ether was used for the extraction.

Stability of ^{14}C-AEG in Human Urine

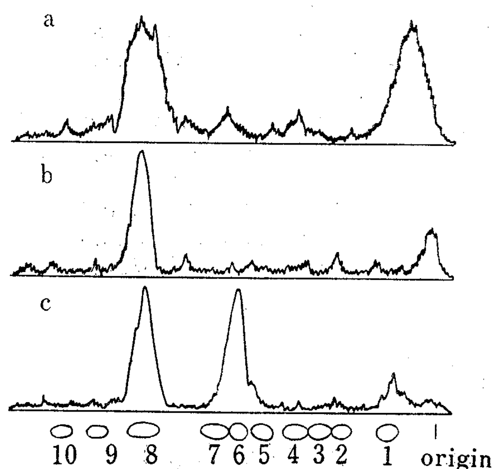

Fig. 1. Radiochromatogram of Steroids liberated from 4-^{14}C-Androstenedione 3-Enol Glucosiduronate

a: after incubation with human urine at 37° for 48 hr
b: after hydrolysis at pH 3.5 for 1 hr
c: after incubation with Ketodase at pH 6.2 for 48 hr

1. 6β-hydroxyandrostenedione and 5β-androstane-3α, 17β-diol 2. 5α-androstane-3α(β), 17β-diol 3. 5β-androstane-3β, 17β-diol 4. testosterone and 3α-hydroxy-5β-androstan-17-one 5. 17β-hydroxy-5β-androstan-3-one 6. androsterone, 3β-hydroxy-5α-androstan-17-one, and 6-oxoandrostenedione 7. 17β-hydroxy-5α-androstan-3-one 8. androstenedione 9. 5β-androstane-3, 17-dione 10. 5α-androstane-3,17-dione

In order to obtain information concerning the stability of the enol glucosiduronate, ^{14}C-AEG was incubated at 37° with human urine, which was filtered with Millipore filter to eliminate bacterial contamination. After 48 and 120 hr, 49 and 69% of the radioactivity were extractable with ether, respectively, indicating that ^{14}C-AEG was cleaved in the urine. The liberated steroids were then examined by TLC. About 48% of the radioactivity appeared in the androstenedione fraction, while 39—48% appeared near the origin (Fig. 1a). For comparison, ^{14}C-androstenedione was also incubated for 120 hr with the same urine. Ninety-seven percent of the radioactivity were extracted with ether and was found to be unchanged ^{14}C-androstenedione, when examined by TLC. Thus, it became quite apparent that ^{14}C-AEG was hydrolyzed in the urine non-enzymatically and/or enzymatically to yield appreciable amount of artifacts (polar steroids) besides androstenedione. These results strongly suggest the difficulty encountered in the isolation and identification procedures of the enol glucosiduronate from the urine.

Stability of ^{14}C-AEG in Buffer Solutions

According to Wakabayashi, *et al.*[8] AEG was moderately stable in neutral and basic media, while rapidly underwent non-enzymic cleavage at acidic pH. They estimated the hydrolysis of AEG by determining both free and conjugated glucuronic acid after incubation at 37° for 1 hr, but did not analyze the liberated steroids. Formation of artifacts by incubation of ^{14}C-AEG with human urine as described above made us to investigate the hydrolysis of ^{14}C-AEG by examining the liberated labeled steroids, which were extractable with ether. The results are shown in Fig. 2. After 1 hr incubation, hydrolysis rates of ^{14}C-AEG were about halves of those reported by Wakabayashi, *et al.* These discrepancies might be attributed to the different methods employed for the determination of the hydrolysis. One possibility is that the enol glucosiduronate might be in part estimated as free glucuronic acid due to its instability. The other possibility is the formation of liberated polar steroids which may not be extractable with ether. However, the latter possibility seems unlikely, because 94 and 83% of the radioactivity were extracted with ether after 24 hr incubation at pH 3.5 and 4.5, respectively. Irrespective of these discrepancies, similar tendencies were observed for the hydrolysis of the enol glucosiduronate. Apparent

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correlation was observed between hydrolysis rate (%) and pH: 94% (pH 3.5, after 24 hr); 83% (pH 4.5, 48 hr); 78% (pH 5.2, 120 hr); 34% (pH 6.2, 120 hr); 15% (pH 7.0, 144 hr); 11% (pH 8.0, 144 hr). After incubation of ^{14}C-AEG at pH 3.5 for 24 hr, the liberated steroids were examined by TLC. Eighty-eight percent of the radioactivity was in androstenedione fraction, while 9% was polar steroids as shown in Fig. 1b.

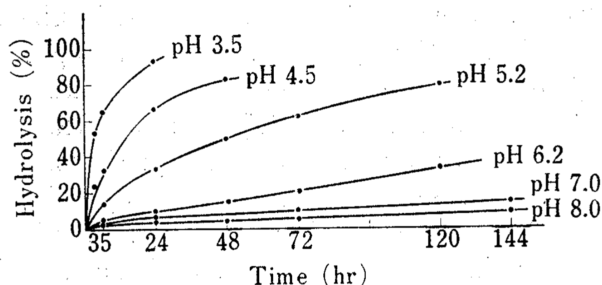

Fig. 2. Hydrolysis of 4-^{14}C-Androstenedione 3-Enol Glucosiduronate in Buffer Solutions at 37°

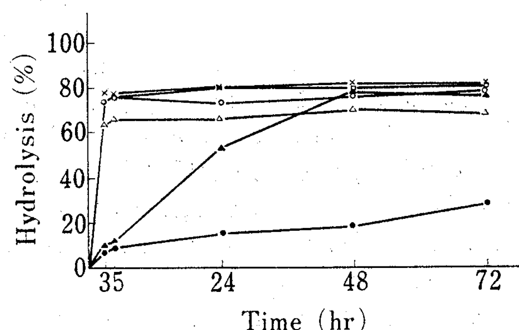

Fig. 3. Hydrolysis of 4-^{14}C-Androstenedione 3-Enol Glucosiduronate by β-Glucuronidase

—○—: Ketodase (beef liver)
—□—: bacterial β-glucuronidase
—×—: bacterial β-glucuronidase with D-glucaro-(1→4)-lactone
—△—: *Helix pomatia* β-glucuronidase
—▲—: *Helix pomatia* β-glucuronidase with D-glucaro-(1→4)-lactone
—●—: without enzyme

Solvolysis of ^{14}C-AEG

Usually, steroid glucosiduronates were not hydrolyzed by mild solvolytic conditions,[17] which were widely employed for the hydrolysis of steroid sulfates. Thus, solvolysis of ^{14}C-AEG was examined by treating with acidic ethyl acetate.[14] After solvolysis, 94% of the radioactivity was extracted with ethyl acetate. Subsequent examination of the liberated steroids by TLC revealed that 87% of the radioactivity appeared in androstenedione fraction, 11% being in 5β-androstane-3α,17β-diol and 6β-hydroxyandrostenedione fraction. Thus, it became quite clear that ^{14}C-AEG was hydrolyzed in the same way as steroid sulfates.

Stability of ^{14}C-AEG during the Isolation Procedures

From the preceding results, the instability of ^{14}C-AEG was clearly demonstrated. The question should be raised whether we could actually isolate most of the enol glucosiduronate intact from the biological materials. Therefore, the stability of ^{14}C-AEG was examined by subjecting to isolation procedures, which were used to study the *in vitro* metabolism of testosterone and its conjugates.[11] To the solution of ^{14}C-AEG in Tris buffer (pH 7.2) was added methylene dichloride and methanol. After evaporation of the solvent, the residue was dissolved in water and extracted with ether. Ninety-seven percent of the radioactivity remained in the aqueous layer, indicating that little hydrolysis occurred by this procedure. The aqueous fraction was passed through a XAD-2 column. The radioactivity was quantitatively recovered in methanol effluent, which was subsequently subjected to solvolysis. Ninety-eight percent of the radioactivity appeared in ethyl acetate fraction. TLC examination of the ethyl acetate fraction gave the following results: androstenedione (87%), 5β-androstane-3α,17β-diol and 6β-hydroxyandrostenedione fraction (6%). Thus, ^{14}C-AEG was fairly stable under the isolation procedures employed by us. The buffer solution certainly played a role in stabilizing

17) H.L. Bradlow, "Chemical and Biological Aspects of Steroids Conjugation," ed. by S. Bernstein and S. Solomon, Springer-Verlag, New York, 1970, p. 131.

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the conjugate. When ^{14}C-AEG was extracted without buffer solution, evaporation of the methanol extract resulted in 16% hydrolysis.

Hydrolysis of ^{14}C-AEG with β-Glucuronidase Preparations

Hydrolysis of the glucosiduronate was usually carried out at acidic pH such as 4.5 or 5.2. However, double pH optima (pH 4.5 and 6.0) for the hydrolysis of the enol glucosiduronate were reported by Wakabayashi, *et al.*[8] Therefore, the hydrolysis of ^{14}C-AEG was examined at pH 6.2 by incubation with Ketodase, β-glucuronidase preparation from *Helix pomatia*, and bacterial β-glucuronidase, because of the considerable non-enzymic hydrolysis of ^{14}C-AEG at acidic pH. The results are shown in Fig. 3. After 3 hr incubation, non-enzymic hydrolysis of ^{14}C-AEG in the same buffer solution was about 10%, while β-glucuronidase preparations hydrolyzed 64—71% of ^{14}C-AEG. However, prolonged incubation did not give quantitative hydrolysis. Even after 72 hr incubation, 20—30% of the radioactivity were not extracted with ether. Steroid glucosiduronates were usually hydrolyzed more than 90% by incubation with Ketodase for 48 hr at pH 5.2.[11] In fact, Ketodase hydrolysis of ^{14}C-AEG at pH 5.2 for 48 hr resulted in 87%. Thus, the incomplete hydrolysis of ^{14}C-AEG might be ascribable to the inactivation of β-glucuronidase or to the formation of polar steroids, which might not be extractable with ether. Next, effect of a powerful β-glucuronidase inhibitor, D-glucaro-(1→4)-lactone,[18] on the hydrolysis of ^{14}C-AEG was investigated, by incubation with *Helix pomatia* and bacterial β-glucuronidase. The addition of the inhibitor (3.45×10^{-3}M) to the incubation media inhibited the hydrolysis with *Helix pomatia* β-glucuronidase, while the same inhibitor had little effect on the hydrolysis with bacterial β-glucuronidase as indicated in Fig. 3. The weak inhibitory action of D-glucaro-(1→4)-lactone upon bacterial β-glucuronidase was reported previously.[19] With *Helix pomatia* β-glucuronidase the inhibition of hydrolysis was complete up to 5 hr incubation, considering the non-enzymic hydrolysis (12%) at pH 6.2. However, marked hydrolysis (53%) occurred after 24 hr incubation with the inhibitor, in comparison with non-enzymic hydrolysis (21%) and enzymic hydrolysis (66%). Prolonged incubation might result in the hydrolysis of the lactone ring of the inhibitor to diminish its inhibitory action.

TLC examination of the liberated steroids, which were obtained by Ketodase hydrolysis at pH 5.2 and 6.2 for 48 hr, revealed the formation of polar steroids besides androstenedione (Fig. 1c): androstenedione fraction (47% at pH 5.2, 40% at pH 6.2), androsterone and 6-oxoandrostenedione fraction (30%, 33%), 5β-androstane-3α,17β-diol and 6β-hydroxyandrostenedione fraction (16%, 23%). The polar steroids should be formed by some non-enzymic mechanisms, since Ketodase was widely used for the hydrolysis of steroid glucosiduronates without any structural changes in the steroid moieties. Incubation of ^{14}C-androstenedione with Ketodase at pH 5.2 resulted in the quantitative recovery of ^{14}C-androstenedione, examined by TLC.

Identification of 6β-Hydroxyandrostenedione

Chemical reactions of steroid 3,5-dien enol ethers were well documented.[20] Electrophilic or radical reagents attack preferentially at C-6 position to produce 6β-substituted 4-en-3-ones. 6β-Hydroxy-4-en-3-ones were prepared from enol ethers of the corresponding 4-en-3-ones by peracid oxidation[21] and by auto-oxidation (light or radical generators).[22] In the paper

18) G.A. Levvy and C.A. Marsh, "Advances in Carbohydrate Chemistry," Vol. 14, ed. by M.L. Wolfrom, Academic Press, New York, 1959, p. 381.
19) C.A. Marsh, *Biochem. J.*, **59**, 375 (1955).
20) D.N. Kirk and M.P. Hartshorn, "Steroid Reaction Mechanisms," Elsevier Publishing Co., New York, 1968, p. 183.
21) J.P. Dusza, J.P. Joseph, and S. Bernstein, *J. Org. Chem.*, **27**, 4046 (1962).
22) R. Gardi and A. Lusignani, *J. Org. Chem.*, **32**, 2647 (1967).

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dealing with the *in vivo* metabolism of androstenedione 3-alkyl enol ether in man, Ercoli[23] briefly mentioned an extraordinary ability of the 3,5-dien-3-enol ether to give the corresponding 6-hydroxy-4-en-3-one by auto-oxidation. Therefore, 6β-hydroxyandrostenedione was one of the probable artifacts produced during the hydrolysis of ^{14}C-AEG. Actually, TLC examination of the liberated steroids suggested the formation of such compound as shown above. The identification of 6β-hydroxyandrostenedione was carried out on the corresponding fraction obtained by incubation with Ketodase at pH 5.2 and 6.2 for 48 hr. Analyses by reverse isotope dilution method demonstrated that purity of 6β-hydroxyandrostenedione in the fraction was about 50%, as indicated in Table I. Another less polar steroid fraction showed a chromatographic mobility similar to androst-4-ene-3,6,17-trione. A portion of this fraction was reduced with sodium borohydride and the product was examined by TLC with solvent S-4. Sixty-one percent of the radioactivity appeared in androst-4-ene-3β,6β,17β-triol fraction. However, recrystallization of the trione fraction with the authentic steroid did not give a constant specific activity.

Table I. Identification by Recrystallization of 6β-Hydroxyandrostenedione obtained by Incubation of 4-^{14}C-Androstenedione 3-Enol Glucosiduronate with Ketodase at pH 6.2 and pH 5.2

No.	Calculated (dpm/mg)	Recrystallization 1st		2nd		3rd		4th		5th	
		Solvent[a]	Specific activity (dpm/mg)	Solvent	Specific activity (dpm/mg)	Solvent	Specific activity (dpm/mg)	Solvent	Specific activity (dpm/mg)	Solvent	Specific activity (dpm/mg)
1[b]	746	B	431	C	335	D	368	B	381		
2[c]	2220	B	1640	C	1340	D	1260	B	1200	C	1190

a) *cf.* "Material and Method" in the text
b) 6β-hydroxyandrostenedione fraction obtained by incubation at pH 6.2
c) 6β-hydroxyandrostenedione fraction obtained by incubation at pH 5.2

From the present study, it became quite apparent that ^{14}C-AEG could undergo non-enzymic transformations, which might be erroneously interpreted as enzymic ones. In order to establish the biochemistry of the steroid enol glucosiduronate, cautious investigation should be required with due regard to its chemical instability.

Acknowledgement The authors express their gratitude to Prof. M. Tomoeda of Kanazawa University for the measurement of mass spectrum.

23) A. Ercoli, "Androgens in Normal and Pathological Conditions," ed. by A. Vermeulen and D. Exley, Excerpta Medica, Amsterdam, 1966, p. 213.

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