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In Vitro Reduction of 16α-Chloroestrone by Rat Liver[1]

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As a series of the metabolic studies on the steroidal lipid-shifting drugs biotransformation of 16α-chloroestrone with the rat liver preparations has been investigated. The substrate was reduced to the 17β-hydroxyl derivative, when incubated with microsomes, but not with the 105000×*g* supernatant. In contrast, estrone was converted into estradiol with either of these enzyme preparations. The yield of 16α-chloroestradiol formed from 16α-chloroestrone by incubation with microsomes decreased with an increasing amount of added estrone indicating the occurrence of competitive inhibition. The properties of 17β-hydroxysteroid dehydrogenase involving 16α-chloroestrone have been described.

16α-Chloroestrone methyl ether is used for the clinical states associated with hypercholesterolemia as a lipid-shifting drug.[3] In the preceding papers of this series we reported the isolation and characterization of the urinary metabolites in the rabbit following oral administration of this drug.[4] Introduction of halogen into the steroid nucleus enhances occasionally the physiological activity inherent to the mother compound. As for 16α-chloroestone methyl ether the metabolic significance of the chloro substituent still remains unclear. The present paper describes enzymatic reduction of 16α-chloroestrone with the rat liver preparations in comparison with that of estrone.

In this study 16α-chloroestrone was employed as a substrate, since estrogen 3-methyl ethers undergo facile O-demethylation in the living animals.[5,6] Although the preparation of this compound has been already disclosed,[7] an alternative synthetic route suitable for the labeled compound was developed. The Δ^{16}-enol acetate, readily obtainable from estrone benzyl ether, was treated with a calculated amount of chlorine under the non-enolizing conditions to yield the 16α-chloro derivative. Subsequent removal of the protecting group at C-3 was effected by hydrogenolysis over palladium-on-charcoal providing the desired 16α-chloroestrone in a satisfactory yield.

As a preliminary experiment 16α-chloroestrone-6,7-^{3}H and estrone-6,7-^{3}H were incubated with the rat liver preparations in the presence of both nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH), respectively. The thin-layer chromatograms of the products formed from each substrate with the subcellular fractions are illustrated in Fig. 1. 16α-Chloroestrone was reduced to the corresponding 17β-hydroxyl derivative with microsomes, but not with the 105000×*g* supernatant. In contrast, estrone was converted into estradiol with either of these two enzyme preparations. The structures of the reduction products could be unequivocally characterized by means of thin-layer (TLC) and gas-liquid chromatography (GLC). Requirement of the cofactor

1) Part CVI of "Studies on Steroids" by T. Nambara; Part CV: H. Hosoda, K. Yamashita, and T. Nambara, *Chem. Pharm. Bull.* (Tokyo), **23**, 3141 (1975). In this paper the following trivial names are used: estrone, 3-hydroxyestra-1,3,5(10)-trien-17-one; estradiol, estra-1,3,5(10)-triene-3,17β-diol.
2) Location: *Aobayama, Sendai.*
3) G.P. Mueller, W.F. Johns, D.L. Cook, and R.A. Edgren, *J. Am. Chem. Soc.*, **80**, 1769 (1958).
4) T. Nambara, M. Nokubo, and Y.H. Bae, *Chem. Pharm. Bull.* (Tokyo), **19**, 2096 (1971); T. Nambara, Y.H. Bae, and M. Nokubo, *J. Chromatog.*, **60**, 418 (1971).
5) M.T. Abdel-Aziz and K.I.H. Williams, *Steroids*, **13**, 809 (1969).
6) T. Nambara, Y.H. Bae, and M. Nokubo, *Yakugaku Zasshi*, **92**, 1157 (1972).
7) G.P. Mueller, U.S. Patent 2855411 [*C. A.*, **53**, 8205 (1959)].

for 17β-hydroxysteroid dehydrogenase was examined with 16α-chloroestrone and estrone. The amount of the 17β-hydroxyl compound produced from each substrate was determined by GLC. The results hereby obtained are shown in Fig. 2. There was seen no significant difference in the yield between NADH and NADPH, when the soluble fraction was employed as an enzyme source. With the microsomal fraction, however, NADH was found to be a much more favorable cofactor than NADPH.

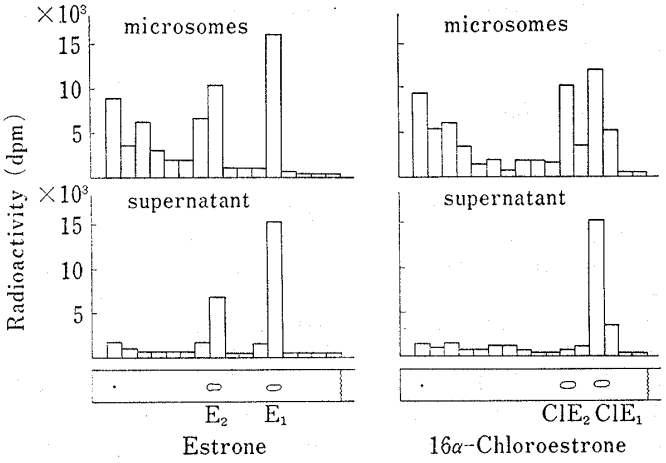

Fig. 1. Thin-Layer Chromatograms of Incubation Products formed from 16α-Chloroestrone-6,7-^{3}H and Estrone-6,7-^{3}H with Rat Liver Preparations

E_1: estrone, E_2: estradiol, ClE_1: 16α-chloroestrone, ClE_2: 16α-chloroestradiol, solvent system: benzene–AcOEt (5:1). adsorbent: silica gel HF (E.Merck AG) Conditions for incubation were as given in the experimental section.

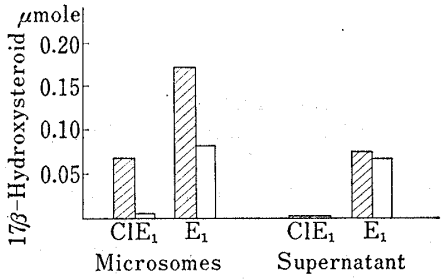

Fig. 2. Effect of Cofactor on Reduction of 16α-Chloroestrone and Estrone with Rat Liver Preparations

▨: NADH, □: NADPH
E_1: estrone, ClE_1: 16α-chloroestrone
Conditions for incubation were as given in the experimental section.

In order to clarify the properties of microsomal 17β-hydroxysteroid dehydrogenase involving 16α-chloroestrone the effect of estrone on enzymatic reduction was then examined. As can be seen in Fig. 3 the yield of 16α-chloroestradiol decreased with an increasing amount of estrone. In this experiment the amount of estradiol yielded increased in proportion to that of estrone added. These results are indicative of estrone being an efficient inhibitor of 16α-chloroestrone reduction with microsomes.

There have been already published several papers dealing with purification and properties of 17β-hydroxysteroid dehydrogenases obtained from the mammalian tissues.[8–12] The present studies revealed that the different types of dehydrogenases are localized depending upon the subcellular fraction. It is of particular interest that the supernatant fraction is lacking in the enzyme activity for reduction of 16α-chloroestrone. Jarabak, *et al.* and Pons,

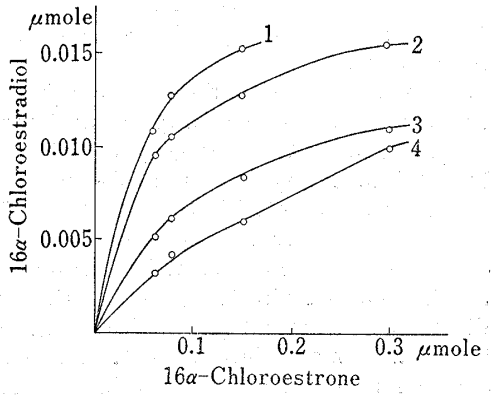

Fig. 3. Effects of Estrone on Reduction of 16α-Chloroestrone with Rat Liver Microsomes

added amounts of estrone 1: none, 2: 0.05, 3: 0.1, 4: 0.2 μmole. Each point represents the mean value of three determinations. Conditions for incubation were as given in the experimental section.

8) J. Jarabak and G.H. Sack, Jr., *Biochemistry*, **8**, 2203 (1969).
9) H.J. Karavolas, M.L. Baedecker, and L.L. Engel, *J. Biol. Chem.*, **245**, 4948 (1970).
10) M.P. Kautsky and D.D. Hagerman, *J. Biol. Chem.*, **245**, 1978 (1970).
11) J.C. Nicolas, M. Pons, B. Descomps, and A. Crastes de Paulet, *FEBS Lett.*, **23**, 175 (1972).
12) H. Inano and B. Tamaoki, *Eur. J. Biochem.*, **44**, 13 (1974).

et al. reported that dehydrogenation of estradiol with the human placental cytosol was markedly interfered by the presence of 16,16-difluoroestrone[8] and 16α-iodoestrone acetate.[13] These findings together imply that the existence of halogen at C-16 would be related to the difficulties with which the 17-ketone is enzymatically reduced to the 17β-hydroxyl compound with the rat liver supernatant fraction. The interference may possibly be due to the irreversible binding to the enzyme with active halogen.

Whether or not the lack of 17β-hydroxysteroid dehydrogenase activity in the soluble fraction is associated with the physiological activity of 16α-chloroestrone methyl ether will be the subject to be investigated in the future.

Experimental

Syntheses of Substrates[14]

16α-Chloroestrone Benzyl Ether——To a stirred solution of 3-benzyloxyestra-1,3,5(10),16-tetraen-17-ol acetate[15] (500 mg) in CCl_4 (25 ml) containing anhydrous K_2CO_3 (600 mg) was added dropwise a calculated amount of Cl_2 dissolved in CCl_4 under ice-cooling. The precipitate was removed by filtration and washed with CCl_4. The filtrate and washings were combined, washed with 5% $Na_2S_2O_3$ and H_2O, dried over anhydrous Na_2SO_4, and evaporated. Recrystallization from MeOH gave 16α-chloroestrone benzyl ether (280 mg) as colorless needles. mp 172—174°. $[\alpha]_D^{14}$ +140° (c=0.24, $CHCl_3$). *Anal.* Calcd. for $C_{25}H_{27}O_2Cl$: C, 76.03; H, 6.89. Found: C, 75.85; H, 6.87. NMR (5% solution in $CDCl_3$) δ: 0.94 (3H, s, 18-CH_3), 4.38 (1H, t, J=3.8 Hz, 16β-H), 4.99 (2H, s, 3-$OC\underline{H}_2C_6H_5$), 6.64—7.20 (3H, aromatic), 7.33 (5H, m, 3-$OCH_2C_6\underline{H}_5$).

16α-Chloroestrone——A solution of 16α-chloroestrone benzyl ether (200 mg) in EtOH (10 ml)–AcOEt (10 ml) was shaken with 10% Pd/C (150 mg) under a stream of H_2 gas for 2 hr. After removal of the catalyst by filtration the filtrate was concentrated *in vacuo* and recrystallized from MeOH to give 16α-chloroestrone (80 mg) as colorless needles. mp 236—238°. NMR (2% solution in $CDCl_3$) δ: 0.97 (3H, s, 18-CH_3), 4.45 (1H, t, J=4.5 Hz, 16β-H), 4.82 (1H, s, 3-OH), 6.55—7.22 (3H, aromatic). Mueller, *et al.*[7] prepared this compound by the different method (lit. mp 238.5—240°).

16α-Chloroestrone-6,7-^{3}H——Prepared from estrone-6,7-^{3}H (200 μCi, 50 μmoles) in the same manner as described with the non-labeled estrone. Total yield 22%. The radiochemical purity was determined by the reverse isotope dilution method. The product was diluted with the carrier and recrystallized from MeOH three times. Calcd.: 6600 dpm/mg. Found: 6500, 6400, 6500 dpm/mg.

Material——NADPH and NADH were obtained from Oriental Yeast Co., Ltd. (Tokyo). Estrone-6,7-^{3}H (48 Ci/mmole) was purchased from the Radiochemical Centre (Amersham, England) and purified by TLC prior to use.

Radioactivity Counting——Counting was performed on a Packard Tri-Carb Model 3380 liquid scintillation counter employing Bray's scintillator.[16]

Thin-Layer Chromatography——TLC was carried out on a silica gel HF (E. Merck AG, Darmstadt) plate by the following systems and *Rf* value was given: TL-I=benzene–AcOEt (5: 1); TL-II=$CHCl_3$–ether (3: 1). 16α-Chloroestradiol: TL-I 0.39; TL-II 0.52. Estradiol: TL-I 0.21; TL-II 0.37.

Gas-Liquid Chromatography——The apparatus used for this work was a Shimadzu Model GC-3BF gas chromatograph equipped with a hydrogen flame ionization detector and a "silanized" spiraled glass tube (3 mm i.d.) packed with 1.5% OV-1 on Chromosorb W (100—120 mesh) (3 m) (GC—I) or 1.5% OV-17 on Shimalite W (60—80 mesh) (2.1 m) (GC—II). Column temperature was 230°, while both the detector and flash heater were maintained at 260°. Nitrogen was used as a carrier gas at flow-rates of 21 ml/min for GC—I and 50 ml/min for GC—II. Trifluoroacetyl derivatives were prepared according to the procedure of VandenHeuvel, *et al.*[17] Retention time relative to estrone methyl ether (GC—I 10.8 min; GC—II 11.2 min), t_R, was given. 16α-Chloroestradiol: GC—I 0.90; GC—II 0.43. Estradiol: GC—I 0.66; GC—II 0.26. 16α-Chloroestradiol and estradiol were determined employing estrone methyl ether as an internal standard.

Enzyme Preparation——Male Wistar rats weighing 250 g on the average were sacrificed by decapitation and liver was immediately removed and chilled on ice. All the subsequent procedures were carried out at 0—4°. The tissue was weighed, minced with scissor, transferred into 4-fold volume of an ice-cooled 1.15%

13) M. Pons, J.C. Nicolas, A.M. Boussioux, B. Descomps, and A. Crastes de Paulet, *FEBS Lett.*, **31**, 256 (1973).
14) All melting points were taken on a micro hot-stage apparatus and are uncorrected. Nuclear magnetic resonance spectra were run on a Hitachi Model R-20 spectrometer at 60 MHz employing tetramethylsilane as an internal standard. Abbreviation used s=singlet, t=triplet, and m=multiplet.
15) T. Nambara and K. Imai, *Chem. Pharm. Bull.* (Tokyo), **15**, 1232 (1967).
16) G.S. Bray, *Anal. Biochem.*, **1**, 279 (1960).
17) W.J.A. VandenHeuvel, J. Sjövall, and E.C. Horning, *Biochim. Biophys. Acta*, **48**, 596 (1961).

KCl solution, and homogenized by a Teflon-glass Potter-Elvehjem homogenizer. The homogenate was centrifuged at 9000 × *g* for 30 min and the pellet was removed. The supernatant was again centrifuged at 105000 × *g* for 60 min by a Hitachi Model 40P ultracentrifuge. The supernatant was separated and used for the incubation study (supernatant fraction). The microsomal pellet was resuspended in a 1.15% KCl solution in such a way that 1 ml was equivalent to 200 mg wet weight of rat liver (microsomal fraction).

Incubation Study——i) 16α-Chloroestrone-6,7-^{3}H or estrone-6,7-^{3}H (1 μCi, 0.25 μmole) in propylene glycol (0.1 ml), NADH (5 μmoles), NADPH (5 μmoles), and the microsomal or supernatant fraction (equivalent to 200 mg wet weight of rat liver) were brought to 5 ml with 0.05M Tris–HCl buffer (pH 7.4) and incubated at 37° for 30 min. The incubation mixture was extracted with AcOEt (5 ml × 3). The organic layer was combined, dried over anhydrous Na_2SO_4, and evaporated. The residue was submitted to TLC using benzene–AcOEt (5: 1) as developing solvent. The adsorbent was scraped from the plate in each 1 cm width and submitted to radioactivity counting (Fig. 1).

ii) 16α-Chloroestrone or estrone (0.5 μmole), NADH or NADPH (5 μmoles), and the microsomal or supernatant fraction (equivalent to 200 mg wet weight of rat liver) were brought to 3 ml with 0.05M Tris–HCl buffer (pH 7.4) and incubated at 37° for 30 min. After addition of AcOEt to stop the reaction, estrone methyl ether (25 μg) was added and extracted with AcOEt (3 ml × 3). The organic layer was combined, dried over anhydrous Na_2SO_4, and evaporated. The residue was submitted to preparative TLC using benzene–AcOEt (5: 1) as developing solvent. Elution of the adsorbent corresponding to 16α-chloroestradiol (*Rf* 0.39) or estradiol (*Rf* 0.21) and estrone methyl ether (internal standard) (*Rf* 0.61) with AcOEt and the eluate was submitted to gas chromatographic determination by condition GC—I (Fig. 2).

iii) 16α-Chloroestrone (0.06, 0.075, 0.15, 0.3 μmole), estrone (0, 0.05, 0.1, 0.2 μmole), NADH (2 μmoles), and the microsomal fraction (equivalent to 200 mg wet weight of rat liver) were brought to 3 ml with 0.05M Tris–HCl buffer (pH 7.4) and incubated at 37° for 10 min. The incubation mixture was processed and 16α-chloroestradiol was determined by GLC in the manner as described in ii) (Fig. 3).

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Reaction of Vinylogous Esters with Grignard Reagent[1)]

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With Grignard reagent, five-membered vinylogous esters gave 1,2-addition products in a similar fashion to the cases for six-membered vinylogous esters. On the other hand, the six-membered vinylogous ester substituted with *t*-butyl group at the α-position of carbonyl function also gave only 1,2-addition product. However the five-membered vinylogous ester substituted with hydroxy group at the α-position of methoxy group gave 1,2-addition product and 1,4-addition products.

In these Grignard reactions, the catalytic effect of cuprous chloride was scarcely observed.

In our previous papers, the reactions of some vinylogous esters with lithium aluminum hydride were examined,[3)] that is, six-membered vinylogous esters were reduced to β-alkoxy-α,β-unsaturated alcohols (due to 1,2-addition), on the other hand, five-membered vinylogous esters were reduced to α,β-unsaturated alcohols (due to 1,4-addition followed by 1,2-addition). The vinylogous esters substituted with *t*-butyl or hydroxy groups were reduced to characteteristic products respectively.

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1) A part of this work was presented at the 95th Annual Meeting of Pharmaceutical Society of Japan, Nishinomiya, April, 1975.
2) Location: *Gofuku, Toyama.*
3) K. Matoba and T. Yamazaki, *Yakugaku Zasshi*, **93**, 1406 (1973) and references therein.