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Studies on Metabolism of 3-Deoxysteroids. VII. Migration of Deuterium during Aryl Hydroxylation of 3-Deoxyestrone[1]

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In order to examine the occurrence of "NIH shift" during hydroxylation of the aromatic steroid, 2- and 3-deuterio-3-deoxyestrones (IIIb, V) were synthesized as substrate. After oral administration of these labeled steroids to a rabbit, two principal metabolites, 2-hydroxy-3-deoxyestrone (Ib) and 17α-estradiol, were isolated from the collected urine specimen. Inspection of mass and nuclear magnetic resonance spectra revealed that aryl hydroxylation was accompanied by a migration of deuterium from the initially labeled site to an adjacent position. The direction and extent of isotope migration are listed in Table I.

It has recently been demonstrated that enzymatic hydroxylation of the specifically tritiated and deuterated aromatic substrate is accompanied by a migration of the heavy isotope from the site of hydroxylation to an adjacent position. The name "NIH shift" has been given to this type of hydroxylation-induced intramolecular migration.[3,4] In a preceding paper we reported that hydroxylation takes place at C-2 and C-3 when 3-deoxyestrone is administered to a rabbit.[5] It appeared, therefore, to be of interest to us to examine whether or not the substituent displaced by an entering hydroxyl group would undergo 1,2-shift during hydroxylation of aromatic ring A. The present paper deals with the occurrence of "NIH shift" during *in vivo* hydroxylation of the labeled 3-deoxyestrone.

2-Deuteriosteroid was prepared starting from 2-hydroxy-3-deoxyestradiol (Ia) in three steps. Condensation with 1-phenyl-5-chlorotetrazole in the presence of potassium carbonate[6] gave the 2-(1-phenyl-5-tetrazolyl) ether (II). Catalytic reduction over palladium-on-barium carbonate under a stream of deuterium gas followed by oxidation with Jones reagent[7] furnished desired 2-deuterio-3-deoxyestrone (IIIb). In a similar fashion 3-deuterio-3-deoxyestrone (V) was also synthesized from estrone by way of 3-(1-phenyl-5-tetrazolyl) ether (IV). The quantity of the isotope in these labeled steroids was determined by mass spectral technique. In order to ensure the localization of deuterium at C-3 in V, 3-deuteriosteroid was submitted to Friedel–Crafts reaction with acetyl chloride. As previously reported[8] acetylation readily occurred to give the 2-acetyl derivative, whose nuclear magnetic resonance (NMR) spectrum exhibited two singlets assignable to C-1 and C-4 protons indicating the presence of the label at C-3.

1) A preliminary account of this work has been presented: T. Nambara, M. Numazawa, and S. Akiyama, *Chem. Pharm. Bull.* (Tokyo), **17**, 2394 (1969). This paper constitutes Part XLII of the series entitled "Analytical Chemical Studies on Steroids"; Part XLI: T. Nambara and H. Takahashi, *Chem. Pharm. Bull.* (Tokyo), **18**, 2309 (1970). The following trivial names are used in this report: 3-deoxyestrone=estra-1,3,5(10)-trien-17-one; 3-deoxyestradiol=estra-1,3,5(10)-trien-17β-ol; 17α-estradiol=estra-1,3,5-(10)-triene-3,17α-diol.
2) Location: *Aobayama, Sendai.*
3) G. Guroff, C. Reifsnyder, and J.W. Daly, *Biochem. Biophys. Res. Commun.*, **24**, 720 (1966).
4) G. Guroff, J.W. Daly, D.M. Jerina, J. Renson, S. Udenfriend, and B. Witkop, *Science*, **157**, 1524 (1967).
5) T. Nambara and M. Numazawa, *Chem. Pharm. Bull.* (Tokyo), **17**, 1200 (1969).
6) W.J. Musliner and J.W. Gates, Jr., *J. Am. Chem. Soc.*, **88**, 4271 (1966).
7) K. Bowden, I.M. Heilbron, E.R.H. Jones, and B.C.L. Weedon, *J. Chem. Soc.*, **1946**, 39.
8) T. Nambara, S. Honma, and S. Akiyama, *Chem. Pharm. Bull.* (Tokyo), **18**, 474 (1970).

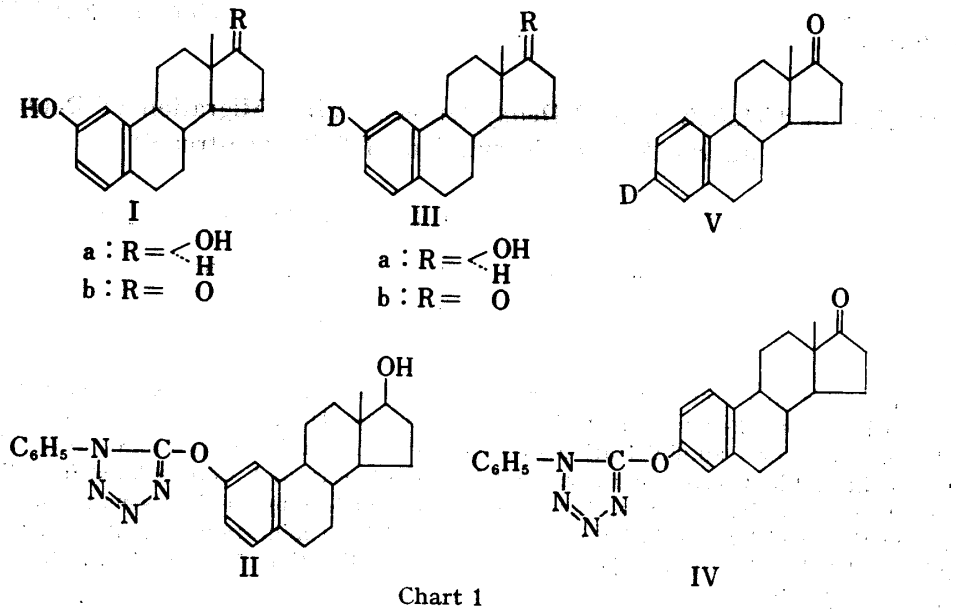

Chart 1

A suspension of IIIb in Tween 80 was orally given to a rabbit. The urine collected for the following 72 hr after administration of the steroid was processed with beef-liver β-glucuronidase and the hydrolyzate was in turn subjected to solvolysis. Isolation of two principal metabolites, 2-hydroxy-3-deoxyestrone (Ib) and 17α-estradiol, was achieved by column chromatography on alumina. Another experiment with use of V as substrate was then carried out. Administration of the labeled steroid and subsequent treatment in the manner as mentioned above gave the desired phenolic metabolites.

Result and Discussion

The retention and locality of the labeled deuterium in the isolated metabolites were determined by inspection of molecular ion peak in mass spectra and of aromatic proton region in NMR spectra. The retention of the isotope in 2- and 3-hydroxylated metabolites formed from two substrates is presented in Table I.

Table I. Retention of Deuterium in the Phenolic Metabolites formed from Deuterated 3-Deoxyestrone by *in vivo* Hydroxylation in Rabbit

Substrate	Metabolite		Retention of deuterium (%)
2-Deuterio-3-deoxyestrone	2-hydroxy-3-deoxyestrone	C-3	47
	17α-estradiol	C-2	81
3-Deuterio-3-deoxyestrone	2-hydroxy-3-deoxyestrone	C-3	81
	17-αestradiol	C-2	*ca.* 50
		C-4	*ca.* 20

Inspection of the molecular ion peak of the metabolites derived from 2-deuteriosteroid (IIIb) revealed that the intramolecular shift of the label did evidently take place with 47% retention during C-2 hydroxylation. In addition NMR spectrum of the aromatic proton region[8-10] was indicative of the localization of deuterium at C-3 rather than at C-1. As

9) J. Fishman and J.S. Liang, *Tetrahedron*, **24**, 2199 (1968).
10) T. Nambara and S. Honma, *Chem. Pharm. Bull.* (Tokyo), **18**, 1191 (1970).

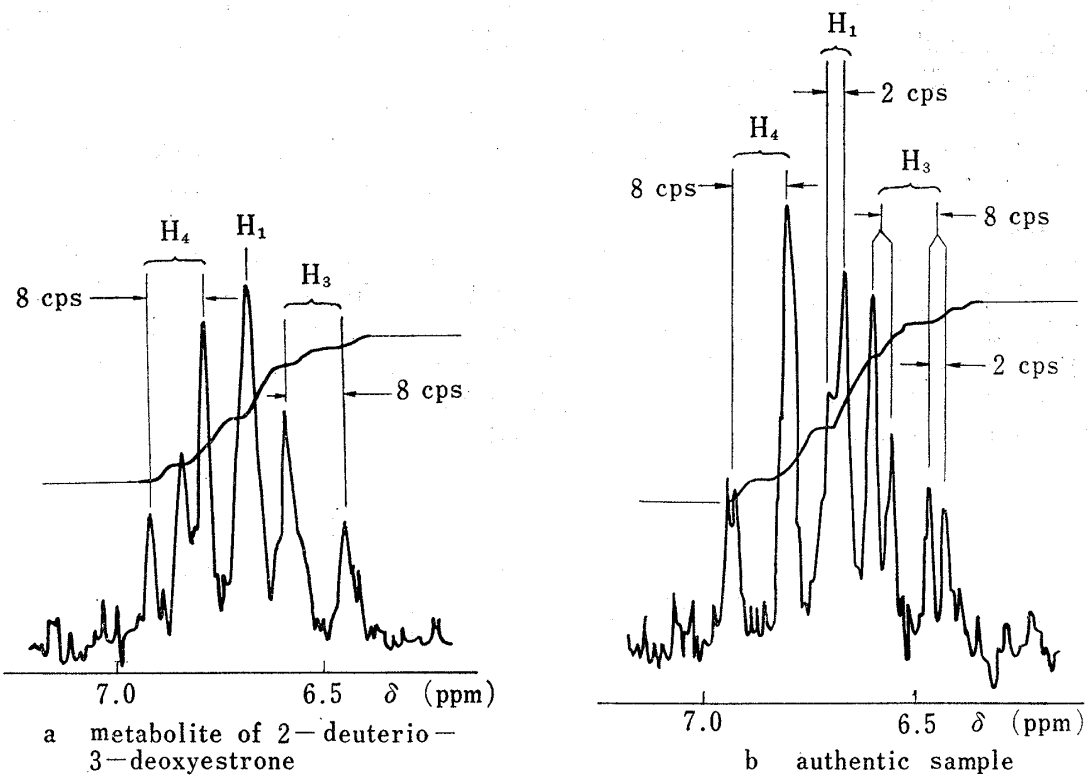

Fig. 1. NMR Spectra of 2-Hydroxy-3-deoxyestrone in DMSO

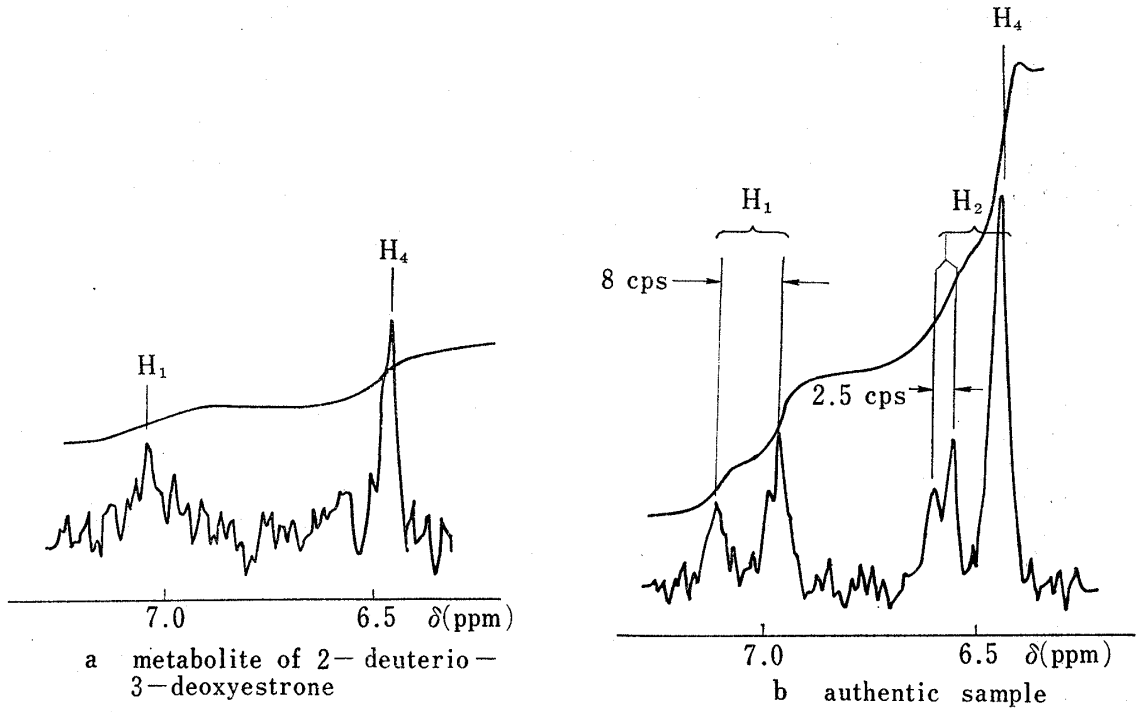

Fig. 2. NMR Spectra of 17α-Estradiol in Acetone

might be expected C-3 hydroxylation actually exerted no marked influence on original deuterium at C-2 (see Fig. 1 and 2).

With regard to the metabolites of 3-deuterio-3-deoxyestrone (V) total deuterium in 17α-estradiol was found to be 74% on the basis of the mass spectrum. The ratio of the isotope distribution between two positions adjacent to an introduced hydroxyl group was roughly

estimated to be 5 to 2 by measuring the intensity of aromatic proton signal. As was shown with 2-deuterated substrate, C-2 hydroxylation did not substantially affect the initial heavy isotope at the adjacent position.

It has been proved that the enzyme-catalyzed aryl hydroxylation of 2- and 3-deuterio-3-deoxyestrones led to transfer of the labeled isotope to the position next to an entering oxygen. To the best of our knowledge this appears to be the first example of "NIH shift" in the field of steroids.

Conney, *et al.*[11-13] suggested that there are many similarities between oxidative drug-metabolizing enzyme and steroid hydroxylase, and the oxidation of drugs and steroids may be catalyzed by the same enzyme system. Moreover it is known that microsomal P-450 is an activator of molecular oxygen in the oxidation of drugs and steroid hormones.[14] The present results indicating the close similarity in hydroxylation mechanism between aromatic steroids and drugs do support the previous findings.

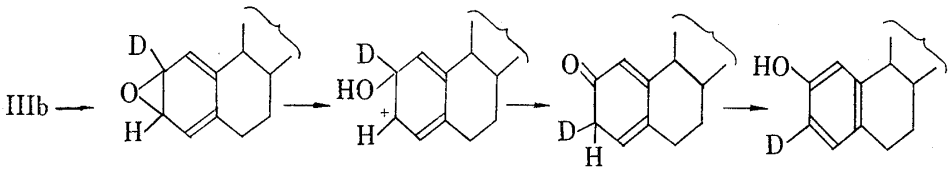

Chart 2

It is also to be noted that the C-2 labeled deuterium migrated selectively to C-3 during hydroxylation at C-2. Recently, Jerina, *et al.*[15,16] reported that the arene oxide may be a possible intermediate for enzymatic hydroxylation of the aromatic substrate. In view of this attractive mechanism the occurrence of 2- and 3-oxygenated estratrienes suggests the participation of the arene 2,3-oxide as a potential intermediate common to both biotransformation products. The opening of the oxido ring at C-3 results in formation of 2-oxygenated cationoid where the positive charge is localized at C-3 probably due to the relative stability. The contribution of this cationoid as a major species is responsible for the preferential migration of the labeled isotope to C-3 rather than to C-1. It is hoped that further studies in progress in this laboratory will provide more precise knowledge on this problem.

Experimental[17]

Synthesis of Substrates

2-(1-Phenyl-5-tetrazolyloxy)estra-1,3,5(10)-trien-17β-ol (II)——To a solution of 1-phenyl-5-chlorotetrazole (1 g) and 2-hydroxy-3-deoxyestradiol (Ia) (960 mg) in acetone (50 ml) was added anhydrous K_2CO_3 (1.5 g), and the resulting solution was refluxed for 22 hr. After removal of the precipitate by filtration the filtrate was concentrated to give a crystalline product. Recrystallization from aq. acetone gave II (1.38 g) as colorless needles. mp 140—141°. $[\alpha]_D^{24}$ +83.4° (c=0.16, $CHCl_3$). *Anal.* Calcd. for $C_{25}H_{28}O_2N_4$: C, 72.09; H, 6.78; N, 13.45. Found: C, 72.12; H, 6.55; N, 13.73.

11) A.H. Conney and A. Klutch, *J. Biol. Chem.*, **238**, 1611 (1963).
12) R. Kuntzman, M. Jacobson, K. Schneidman, and A.H. Conney, *J. Pharmacol. Exptl. Therap.*, **146**, 280 (1964).
13) A.H. Conney, W. Levin, M. Ikeda, R. Kuntzman, D.Y. Cooper, and O. Rosenthal, *J. Biol. Chem.*, **243** 3912 (1968).
14) R. Kato, A. Takahashi, and Y. Omori, *Life Sci.*, **7**, 915 (1968), and references quoted therein.
15) D.M. Jerina, J.W. Daly, and B. Witkop, *J. Am. Chem. Soc.*, **90**, 6523 (1968).
16) D.M. Jerina, J.W. Daly, B. Witkop, P. Zaltzman-Nierenberg, and S. Udenfriend, *J. Am. Chem. Soc.*, **90**, 6525 (1968).
17) All melting points were taken on a micro hot-stage apparatus and are uncorrected. NMR spectra were recorded on Varian Model A-60 spectrometer using tetramethylsilane as an internal standard (TMS=0.0 ppm). Mass spectral measurements were run on Hitachi Model RMU-6D spectrometer.

2-Deuterioestra-1,3,5(10)-trien-17-one (2-Deuterio-3-deoxyestrone) (IIIb)——A solution of II (1.3 g) in benzene (20 ml) was shaken with $Pd/BaCO_3$ (2.6 g) under a stream of D_2 gas for 8 days. After removal of catalyst by filtration the filtrate was concentrated to give a crystalline product. The crude product thus obtained was chromatographed on Al_2O_3 (20 g). Elution with hexane–benzene (1:1) gave 2-deuterioestra-1,3,5(10)-trien-17β-ol (IIIa). The eluate was homogeneous according to thin-layer chromatography (TLC) and therefore submitted to further elaboration without purification. To a solution of IIIa in acetone (10 ml) was added Jones reagent (0.9 ml) under ice-cooling, and the solution was allowed to stand for 5 min. The reaction mixture was poured into ice-water and extracted with ether. After usual work-up recrystallization of the crude product from ether gave IIIb (375 mg) as colorless needles. mp 141—142°. Mixed melting point on admixture with the authentic sample showed no depression. By mass spectrometry the deuteriosteroid synthesized was calculated to consist of 57% monodeuterio species along with 16% dideuterio and 26% normal 3-deoxyestrone.

3-(1-Phenyl-5-tetrazolyloxy)estra-1,3,5(10)-trien-17-one (IV)——To a solution of 1-phenyl-5-chlorotetrazole (1 g) and estrone (1 g) in acetone (60 ml) was added anhydrous K_2CO_3 (1.5 g), and the resulting solution was refluxed for 17 hr. After removal of the precipitate by filtration the filtrate was concentrated to give a crystalline product. Recrystallization from aq. acetone gave IV (1.26 g) as colorless needles. mp 204—206°. $[\alpha]_D^{26}$ +102.8° (c=0.13, $CHCl_3$). *Anal.* Calcd. for $C_{25}H_{26}O_2N_4$: C, 72.44; H, 6.32; N, 13.52. Found: C, 72.41; H, 6.11; N, 13.65.

3-Deuterioestra-1,3,5(10)-trien-17-one (3-Deuterio-3-deoxyestrone) (V)——A solution of IV (2 g) in benzene (50 ml) was shaken with $Pd/BaCO_3$ (3.2 g) under a stream of D_2 gas for 10 days. After removal of catalyst by filtration the filtrate was concentrated. The crude product thus obtained was chromatographed on Al_2O_3 (27 g). Elution with hexane–benzene (9:1) and recrystallization of the eluate from ether gave V (1.22 g) as colorless needles. mp 141—142°. Mixed melting point on admixture with the authentic sample showed no depression. By mass spectrometry the deuteriosteroid synthesized was calculated to consist of 58% monodeuterio species with 20% dideuterio and 22% normal 3-deoxyestrone.

Transformation of V into 2-Acetyl-3-deuterio-3-deoxyestradiol Acetate——To a stirred solution of V (75 mg) in MeOH (10 ml) was added $NaBH_4$ (12 mg) and the resulting solution was allowed to stand at room temperature for 20 min. Usual work-up followed by acetylation with Ac_2O (2 ml) and pyridine (2 ml) gave 3-deuterio-3-deoxyestradiol acetate. Anhydrous $AlCl_3$ (200 mg) was dissolved in CS_2 (5 ml) containing AcCl (1 ml) by stirring for 10 min. To this solution was added a solution of 3-deuterio-3-deoxyestradiol acetate in CH_2Cl_2 (5 ml) over a period of 10 min and stirred for 1 hr at room temperature. The reaction mixture was poured into cold 5% HCl and extracted with ether. The organic phase was washed with 5% $NaHCO_3$ and H_2O and dried over anhydrous Na_2SO_4. After evaporation of solvent the crude product obtained was recrystallized from MeOH to give 2-acetyl-3-deuterio-3-deoxyestradiol acetate (38.5 mg) as colorless needles. mp 131—133°. Mixed melting point on admixture with the authentic sample[8] showed no depression. NMR (6% solution in CCl_4) δ: 0.82 (3H, s, 18-CH_3), 2.00 (3H, s, 17-$OCOCH_3$), 2.46 (3H, s, 2-$COCH_3$), 7.03 (1H, s, 4-H), 7.77 (1H, s, 1-H).

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

Administration of 3-Deoxyestrone——In a typical run a single dose of suspension of 2(or 3)-deuterio-3-deoxyestrone (475 mg) in Tween 80 was injected into a stomach through a catheter, and the urine was collected in a bottle containing a few drops of toluene for 72 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 4.8 with 0.1M acetate buffer (10 ml/100 ml of urine) and incubated with beef-liver β-glucuronidase (Tokyo Zōkikagaku, Co.) (500 Fishman U/ml) at 37° for 5 days. The urine was then brought to 2N H_2SO_4 solution with 50% H_2SO_4, saturated with NaCl (20 g/100 ml) and extracted with AcOEt. The organic phases were combined and allowed to stand at 37° for 12 hr. The extract was 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 Metabolites——The gummy substance was submitted to column chromatography on Al_2O_3 (20 g) and eluted with hexane–benzene (3:7), benzene and benzene–ether (1:1), successively. The combined eluate was rechromatographed on Al_2O_3 (20 g), and each 20 ml of effluent was fractionally collected as follows:

Fraction No.	Solvent system	Yield (mg)	Metabolites
1— 7	benzene		
8—18	benzene	38	2-hydroxy-3-deoxyestrone
19—21	benzene	48	17α-estradiol

Recrystallization of the first eluate from aq. MeOH gave 2-hydroxy-3-deoxyestrone as colorless needles. mp 195—199°. The second eluate was submitted to preparative TLC on silica gel HF (E. Merck AG) using benzene–ether (3:1) as developing solvent. Recrystallization of the eluate from MeOH gave 17α-estradiol as colorless needles. mp 229—230°.

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