$Ba(OH)_2$ solution. Although the emission of CO_2 subsided within 30 min in most cases, N-formylation of the reduction product was inperfect under this condition (see Table II). Succeedingly the mixture was heated for additional two hour at 120—125°. After this, the reaction solution was evaporated under reduced pressure to remove TMAF. The residual liquid was distilled under high reduced pressure to give the product, N-alkyl-N-(α-alkylbenzyl)formamide. In all the runs yields of the product are about 90% as shown in Table II. Physical and analytical data of the products and the corresponding free N-alkyl-α-alkylbenzyl-amines are recorded in Table V and VI.

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C-2 Hydroxylation of 2-Deuterioestrogen in the Rat. Lack of "NIH Shift"[1]

TOSHIO NAMBARA, SETSUKO AKIYAMA, and SEIJIRO HONMA

Pharmaceutical Institute, Tohoku University[2]

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In a previous paper the occurrence of "NIH shift" during aromatic ring hydroxylation with 3-deoxyestrone has been reported.[3] Further interest in hydroxylation mechanism prompted us to examine whether or not *in vivo* formation of the catechol estrogen would similarly be accompanied by a migration of the heavy isotope labeled at C-2.

For this purpose an initial project was focused to the preparation of 2-deuterioestrogen as a substrate. First, 2-hydroxyestrone 3-benzyl ether (I) derivable from estrone in several steps[4] was taken as a starting compound. Condensation with 1-phenyl-5-chlorotetrazole in the presence of potassium carbonate[5] provided phenyltetrazolyl ether (II) in satisfactory yield. Hydrogenolysis over palladium-on-barium carbonate with use of deuterium gas proceeded readily to give 2-deuterioestrone (IIIa).

This synthetic route, however, appeared to be of disadvantage in the availability of the starting material. Accordingly, an attempt was then made on the utilization of 2-halo-estrogen for the preparation of the specifically deuterated substrate. Treatment of estradiol with bromine in acetic acid resulted in formation of a mixture of 2- and 4-bromo derivatives in a ratio of *ca.* 1 to 1.[6] The isolation of the desired 2-bromo isomer (IV) could be attained by preparative thin-layer chromatography (TLC) upon multiple development. However, separation of these two positional isomers was a tedious work because of close similarity in their chromatographic behaviors. Therefore, the more suitable method for preparation of 2-halo derivative was explored. When estrone was treated with iodine in the presence of mercuric acetate as catalyst,[7] halogenation occurred selectively at C-2 yielding

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1) This paper constitutes Part XLVII of the series entitled, "Analytical Chemical Studies on Steroids"; Part XLVI: T. Nambara and M. Numazawa, *Chem. Pharm. Bull.* (Tokyo), **19**, 990 (1971). When this work was almost completed we learned a paper dealing with the preparation of C-2 isotope labeled estradiol from 2-bromo derivative and the loss of tritium during *in vivo* hydroxylation at C-2 in man (J. Fishman, H. Guzik, and L. Hellman, *Biochemistry*, **9**, 1593 (1970)).
2) Location: *Aobayama, Sendai.*
3) T. Nambara, M. Numazawa, and S. Akiyama, *Chem. Pharm. Bull.* (Tokyo), **19**, 153 (1971).
4) T. Nambara, S. Honma, and S. Akiyama, *Chem. Pharm. Bull.* (Tokyo), **18**, 474 (1970).
5) W.J. Musliner and J.W. Gates, Jr., *J. Am. Chem. Soc.*, **88**, 4271 (1966).
6) T. Utne, R.B. Jobson, and F.W. Landgraf, *J. Org. Chem.*, **33**, 1654 (1968).
7) A. Hillmann-Elies, G. Hillmann, and U. Schiedt, *Z. Naturforschung* (*B*), **8**, 436 (1953).

2-iodoestrone (Va) as a sole product. The orientation of an introduced halogen was confirmed by inspection of the aromatic proton signal in the nuclear magnetic resonance (NMR) spectra.[4,8] Similar treatment of estradiol gave 2-iodoestradiol (Vb), which proved to be identical with the product derived from Va by sodium borohydride reduction. Upon catalytic hydrogenation over palladium-on-barium carbonate under a stream of deuterium gas 2-haloestradiols were led to 2-deuterioestradiol (IIIb) in reasonable yield. The distribution and quantity of the isotope in this specifically labeled steroid were determined by means of NMR and mass spectral techniques.

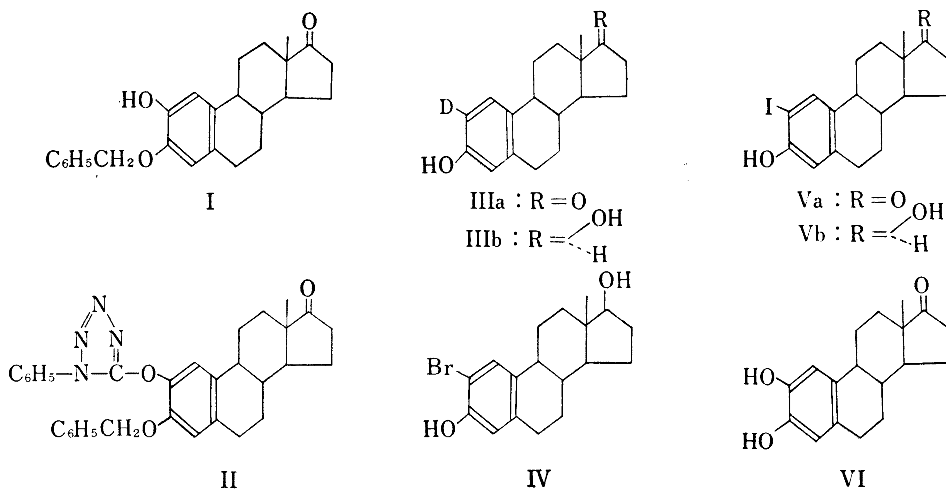

Chart 1

A suspension of IIIb in Tween 80 was intraperitoneally injected to five male rats and the urine was collected for the following four days. The urine specimen was first processed with beef–liver β-glucuronidase in the usual manner. The hydrolyzate was then extracted with ethyl acetate, which in turn was subjected to solvolysis.[9] The gummy substance thus obtained was purified by means of the preparative TLC. Upon multiple development the desired 2-hydroxyestrone (VI) could be isolated in the crystalline state with success.

The retention of the labeled deuterium in the isolated metabolite was determined by inspection of molecular ion peak in mass spectra. It was found that the excreted metabolite lost the label completely, thus demonstrating that the C-2 hydroxylation proceeded without any retention of the heavy isotope. This result is not surprising since the lack of retention of deuterium during *ortho* hydroxylation has already been reported with other instances of catechol formation. The loss of the label can be explained in such a way that hydroxylation proceeds through a quinoid intermediate which would readily release *ortho* hydrogen.[10]

In our previous study it has been observed that the rate of *in vivo* hydroxylation followed by O-methylation at C-2 is more efficient with estrogen sulfate than with estrogen itself in the rat.[11] The lack of "NIH shift" implies that free estrogen rather than its conjugate may probably serve as a substrate for enzymatic hydroxylation leading to the formation of catechol estrogen. Therefore, the enhanced efficiency with which the estrogen sulfate is transformed into the 2-oxygenated metabolite may be ascribable to the improvement of the mem-

8) J. Fishman and J.S. Liang, *Tetrahedron*, **24**, 2199 (1968).
9) S. Burstein and S. Lieberman, *J. Biol. Chem.*, **233**, 331 (1958).
10) J. Daly, G. Guroff, S. Udenfriend, and B. Witkop, *Arch. Biochem. Biophys.*, **122**, 218 (1967); J. Daly, D. Jerina, and B. Witkop, *ibid.*, **128**, 517 (1968).
11) T. Nambara, S. Honma, and K. Kanayama, to be published.

brane permeability upon conjugation with sulfuric acid. Recently the biochemical significance of conjugation, in particular sulfate formation, has become an attractive problem associated with the metabolism of physiologically active substances.[12,13] It is hoped that further work in progress in this laboratory will provide the more precise knowledge on this problem.

Experimental[14]

Synthesis of Substrates

3-Benzyloxy-2-(1-phenyl-5-tetrazolyloxy)estra-1,3,5(10)-trien-17-one (II)——To a stirred solution of 2-hydroxyestrone 3-benzyl ether (I)[4] (213 mg) and 1-phenyl-5-chlorotetrazole (242 mg) in AcOEt (50 ml) was added anhydrous K_2CO_3 (400 mg) and the suspended solution was refluxed for 5.5 hr. After removal of the precipitate by filtration the filtrate was concentrated to give a crystalline product. Recrystallization from aq. MeOH gave II (271 mg) as colorless needles. mp 153—154.5°. $[\alpha]_D^{27}+94.8°$ (c=0.11, $CHCl_3$). *Anal.* Calcd. for $C_{32}H_{32}O_3N_4$: C, 73.82; H, 6.20; N, 10.76. Found: C, 73.63; H, 6.34; N, 10.76.

2-Bromoestra-1,3,5(10)-triene-3,17β-diol (IV), 4-Bromoestra-1,3,5(10)-triene-3,17β-diol——To a solution of estradiol (96 mg) in glacial AcOH (5 ml) was added dropwise a solution of Br_2 (60 mg) in AcOH (4 ml) at 17° and then stirred for 24 hr. On usual work-up the crude product obtained was submitted to preparative TLC using benzene-ether (5:1) as developing solvent. After development was repeated three times, the adsorbent corresponding to the spot (*Rf* 0.45) was eluted with AcOEt. Recrystallization of the eluate from ether and then from EtOH gave IV (8 mg) as colorless needles. mp 196.5—197.5°. $[\alpha]_D^{14}+106.4°$ (c=0.08, $CHCl_3$). *Anal.* Calcd. for $C_{18}H_{23}O_2Br$: C, 61.54; H, 6.60. Found: C, 61.64; H, 6.53. NMR (3% solution in acetone) δ: 0.76 (3H, s, 18-CH_3), 6.67 (1H, s, 4-H), 7.30 (1H, s, 1-H). (Reported[6]: mp 197—198°. $[\alpha]_D^{25}+104°$ (c=0.1, $CHCl_3$)). Elution of the adsorbent corresponding to the spot (*Rf* 0.38) with AcOEt and recrystallization of the eluate from ether and then from EtOH gave 4-bromoestradiol (20 mg) as colorless needles. mp 181.5—183°. $[\alpha]_D^{16}+99.0°$ (c=0.11, $CHCl_3$). *Anal.* Calcd. for $C_{18}H_{23}O_2Br$: C, 61.54; H, 6.60. Found: C, 61.33; H, 6.66. NMR (4% solution in acetone) δ: 0.76 (3H, s, 18-CH_3), 6.71 (1H, d, J=9 cps, 2-H), 7.08 (1H, d, J=9 cps, 1-H). (Reported[6]: mp 213.5—215°. $[\alpha]_D^{25}+43°$ (c=0.1, $CHCl_3$)).

2-Iodoestra-1,3,5 (10)-trien-17-one (Va)——To a solution of estrone (540 mg) in glacial AcOH (10 ml) was added dropwise a solution of Hg $(OAc)_2$ (165 mg) in AcOH (20 ml) saturated with I_2 (520 mg) over a period of 5 min and stirred at 45—55° for 1 hr. After removal of the precipitate by filtration the filtrate was poured into $Na_2S_2O_3$ solution. The precipitate was collected by filtration, washed with H_2O and dried. The crude product was dissolved in benzene and chromatographed on Al_2O_3 (30 g). Elution with benzene and recrystallization of the eluate from AcOH and then from MeOH gave Va (409 mg) as colorless needles. mp 200—205°. $[\alpha]_D^{19}+154.6°$ (c=0.11, $CHCl_3$). *Anal.* Calcd. for $C_{18}H_{21}O_2I$: C, 54.56; H, 5.34. Found: C, 54.78; H, 5.44. NMR (2.5% solution in acetone) δ: 0.90 (3H, s, 18-CH_3), 6.66 (1H, s, 4-H), 7.54 (1H, s, 1-H).

2-Iodoestra-1,3,5 (10)-trien-17β-ol (Vb)——i) To a solution of estradiol (404 mg) in glacial AcOH (20 ml) was added dropwise a solution of $Hg(OAc)_2$ (123 mg) in AcOH (15 ml) saturated with I_2 (440 mg) over a period of 5 min and stirred at 45—55° for 17 hr. After removal of the precipitate by filtration the filtrate was poured into $Na_2S_2O_3$ solution. The precipitate was collected by filtration, washed with H_2O and dried. The crude product was submitted to preparative TLC using hexane-AcOEt (4:1) as developing solvent. After development was repeated five times, the adsorbent corresponding to the spot (*Rf* 0.32) was eluted with AcOEt. Recrystallization of the eluate from benzene and then from acetone gave Vb (56 mg) as colorless needles. mp 161—162°. $[\alpha]_D^{16}+82.0°$ (c=0.12, $CHCl_3$). *Anal.* Calcd. for $C_{18}H_{23}O_2I$: C, 54.28; H, 5.82. Found: C, 54.26; H, 6.19. NMR (5% solution in acetone) δ: 0.78 (3H, s, 18-CH_3), 6.65 (1H, s, 4-H), 7.50 (1H, s, 1-H).

ii) To a solution of Va (15 mg) in MeOH (5 ml) was added portionwise $NaBH_4$ (5 mg) under ice-cooling and the resulting solution was allowed to stand at room temperature for 2 hr. After evaporation of solvent the residue was diluted with AcOEt, washed with H_2O and dried over anhydrous Na_2SO_4. On usual work-up a crystalline product was obtained. Recrystallization from acetone gave Vb (10 mg) as colorless prisms. mp 162—163.5°. Mixed melting point on admixture with the sample obtained in i) showed no depression.

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12) H.E. Hadd and R.T. Blickenstaff, "Conjugates of Steroid Hormons," Academic Press, 1969, p.293.

13) D.S. Layne, "Metabolic Conjugation and Metabolic Hydrolysis,"Vol. 1, ed. by W.H. Fishman, Academic Press, 1970, p. 43.

14) All melting points were taken on a micro hot-stage apparatus and are uncorrected. NMR spectra were recorded on Hitachi Model R-20 spectrometer using tetramethylsilane as an internal standard (TMS=0.0 ppm). Mass spectral measurements were run on Hitachi Model RMU-6D spectrometer. For preparative TLC Silica Gel H (E. Merck AG) was used as an adsorbent.

2-Deuterio-3-hydroxyestra-1,3,5 (10)-trien-17-one (2-Deuterioestrone) (IIIa)——A solution of II (20 mg) in AcOEt (6 ml) was shaken with 5% Pd/$BaCO_3$ (40 mg) under a stream of D_2 gas for 15 hr. After removal of the catalyst by filtration the filtrate was concentrated to give a crystalline product. Recrystallization from acetone gave IIIa (6 mg) as colorless needles. mp 252—254°. Mixed melting point on admixture with the authentic sample showed no depression. By mass spectrometry the deuteriosteroid thus obtained was calculated to consist of 49.8% monodeuterio species along with 24.6% dideuterio and 25.6% normal estrone.

2-Deuterioestra-1,3,5 (10)-triene-3,17β-diol (2-Deuterioestradiol) (IIIb)——A solution of Vb (or IV) (600 mg) in AcOEt (25 ml) was shaken with 5% Pd/$BaCO_3$ (1.4 g) under a stream of D_2 gas for 117 hr. After removal of the catalyst by filtration the filtrate was concentrated to give a crystalline product. Recrystallization from MeOH gave IIIb (554 mg) as colorless needles. mp 178—179°. Mixed melting point on admixture with the authentic sample showed no depression. By mass spectrometry the deuteriosteroid thus obtained was calculated to consist of 72.2% monodeuterio species along with 7.3% dideuterio and 20.5% normal estradiol. NMR (4% solution in acetone) δ: 0.78 (3H, s, 18-CH_3), 6.48 (1H, s, 4-H), 7.03 (1H, s, 1-H).

Animal——Wistar strain male rats weighing *ca.* 200 g were housed in a cage that was designed to minimize fecal contamination of the urine.

Administration of 2-Deuterioestradiol (IIIb)——Each of five rats was intraperitoneally injected with a suspension of IIIb (50 mg) in Tween 80 and again with 34 mg of IIIb on the 2nd day. Urine was collected for the following 4 days after the initial administration.

Hydrolysis with β-Glucuronidase and Solvolysis——The pooled urine (173 ml) was adjusted to pH 4.5 with 50% H_2SO_4 and then to pH 4.6 with 0.1M acetate buffer (20 ml). After addition of Penicillin G (50000 U) the urine was incubated with beef-liver β-glucuronidase (Tokyo Zōkikagaku, Co.) (51900 Fishman U) at 37° for 5 days. The hydrolyzate 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 phase was separated 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 residue was submitted to preparative TLC using benzene–ether (4:1) as developing solvent. The adsorbent corresponding to the spot (*Rf* 0.13) was eluted with $CHCl_3$–MeOH (10:1). The eluate was again chromatographed on TLC plate with use of hexane–$CHCl_3$–AcOH (2:2:1). Elution of the zone corresponding to the spot (*Rf* 0.60) gave 2-hydroxyestrone (VI) as colorless amorphous substance (5 mg). When trimethylsilylated in the usual manner and then submitted to gas chromatography using 3% SE-30 on a support of Chromosorb W (60—80 mesh) as a stationary phase, the metabolite showed the identical retention time (10.0 min) with that of the authentic sample. The gas chromatographic conditions employed were as follows: the column, injection chamber and detector were kept at 215°, 250° and 250°, respectively, and the flow rate of carrier gas (N_2) was 60 ml/min.

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