and dilute sodium carbonate solution and then extracted with ether. The residue (78 mg.) from the washed and dried ether was twice crystallized from ethyl acetate–hexane, yielding small rods, m.p. 216–217°; $[\alpha]^{21}D$ −2° (c 0.578, dioxane); λ_{max}^{alc} 267 mμ (ε 780), 276 (723), 260 sh (595); λ_{max}^{Nujol} 5.63, 5.68, 5.75 (triplet, s), 8.05 (s) μ.

Anal. Calcd. for $C_{26}H_{32}O_8$ (472.5): C, 66.08; H, 6.83. Found: C, 65.76; H, 6.78.

The monoacetate XIVa was also obtained as the sole product when in the preparation of 6β,7β-oxidoestradiol diacetate glacial acetic acid equivalent to the moles of potassium *t*-butoxide was added at the beginning of the work-up.

6β-Methoxy-7α-hydroxyestradiol 3,17-Diacetate(XVa).—6α,-7α-Oxidoestradiol diacetate (32 mg.) was dissolved in reagent methanol (10 ml.) containing 70% perchloric acid (0.04 ml.). After about 10 min. at room temperature, the reaction mixture was neutralized with a saturated solution of sodium bicarbonate. The methanol was removed *in vacuo* and the residue from a washed and dried chloroform extract crystallized on standing in dilute methanol as small needles, m.p. 83–88°; $[\alpha]^{21}D$ +25° (c 0.777, dioxane); λ_{max}^{alc} 267 mμ (ε 620), 275 (580); λ_{max}^{Nujol} 2.95 (m), 5.67 (s), 5.77 (s), 8.07 (s) μ.

Anal. Calcd. for $C_{23}H_{30}O_6$ (402.5): OCH_3, 7.71. Found: OCH_3, 7.79.

When the methanolic mother liquor of 6α,7α-oxidoestradiol diacetate (X) was allowed to stand at room temperature for several days and the solid obtained by removal of the methanol was chromatographed, the main product proved to be 6β-methoxy-7α-hydroxyestradiol 3,17-diacetate. Its melting point, rotation, and spectral data were identical with those of the methanolysis product of the oxide.

Triacetate XVb.—6β-Methoxy-7α-hydroxyestradiol 3,17-diacetate (50 mg.) was acetylated in anhydrous pyridine and acetic anhydride in the usual manner. The reaction product was recrystallized twice from ethyl acetate–hexane yielding fine rods (41 mg.), m.p. 182–183°; $[\alpha]^{21}D$ −9° (c 0.867); λ_{max}^{alc} 267 mμ (ε 613), 275 (564), 263 sh (503); λ_{max}^{Nujol} 5.63 (s), 5.74 (s), 8.04 (s) μ.

Anal. Calcd. for $C_{25}N_{32}O_7$ (444.5): C, 67.55; H, 7.26. Found: C, 67.56; H, 7.37.

6α-Methoxy-7β-Hydroxyestradiol 3,17-Diacetate XVI.—6β,7β-Oxidoestradiol diacetate (43 mg.) was dissolved in reagent methanol (15 ml.) containing 70% perchloric acid (0.053 ml.). After about 10 min. at room temperature, the reaction mixture was neutralized with a saturated solution of sodium bicarbonate. The methanol was removed *in vacuo* and the residue (42 mg.) from the washed and dried chloroform extract was twice crystallized from ethyl acetate–hexane yielding small needles, m.p. 177–178°; $[\alpha]^{20}D$ −18° (c 0.775, dioxane); λ_{max}^{alc} 267 mμ (ε 563), 273 (527); λ_{max}^{KBr} 2.94 (m), 5.57 (s), 5.77 (s), 8.06 (s) μ.

Anal. Calcd. for $C_{23}H_{30}O_6$ (402.5): C, 68.63; H, 7.51; OCH_3, 7.71. Found: C, 68.82; H, 7.79; OCH_3, 7.35.

Acknowledgment.—We wish to thank Dr. J. Iriarte of the Syntex S. A. for making available to us a sample of 6α,7α-dihydroxyestradiol tetracetate (XIIIb) for n.m.r. measurement.

Microbial Hydroxylation of Estrone and Estradiol in the 6β-, 7α-, and 15α-Positions

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Estrone and estradiol have been converted into their 7α- and 15α-hydroxy derivatives by microorganisms of the genera *Glomerella* and *Aspergillus*, and to 6β-estradiol by *Mortierella alpina*. 15α-Hydroxyestrone and 15α-hydroxyestradiol are new compounds of low estrogenic potency.

The stereospecific hydroxylation of steroidal substrates by microbial enzymes has during the past decade developed into one of the most important and widely employed reactions in steroid chemistry. It has been applied to a large variety of substrates particularly those possessing the pregnane and androstane skeleton, and with very few exceptions all available positions of the steroid nucleus have been hydroxylated by this elegant procedure.[2] There is one important class of steroids, however, that is only sparsely represented in the literature in connection with this reaction and these are the phenolic estrogens. Only two reports have appeared,[3,4] both presenting evidence for the 16α-hydroxylation of estrone and estradiol. This limited measure of accomplishment is surely not due to a lack of interest in the hydroxylation of the estrogenic hormones, but, to judge from our own experience, a reflection of the tendency of these phenolic steroids to suffer multiple enzymic attack thereby giving rise to difficultly separable mixtures. The present report, therefore, represents the result of considerable effort involving a large variety of organisms.

The organisms destined for further exploration were selected on the basis of paper chromatographic evidence for complete utilization of the substrate and the formation of substantial amounts of hydroxylated products. They belong to the genera *Glomerella*, *Aspergillus*, and *Mortierella*. The organisms were grown in flasks which were vigorously shaken to promote maximum growth, then transferred to a fresh growth medium, to which the steroid had been added, and allowed to grow under the same conditions. Samples were taken at intervals and analyzed for the degree of conversion by paper chromatography. The fermentations were terminated when all the substrate had disappeared. The steroid metabolites were then recovered from the filtered broth by extraction with chloroform and methyl isobutyl ketone, and the individual components were isolated after chromatography on alumina. It soon became evident that when the time of fermentation was extended from 2 to 7 days the organisms, in addition to hydroxylating estrone, also caused partial reduction of the 17-keto group, thus producing the corresponding hydroxylated estradiol derivatives. The reverse was true when estradiol was used as a substrate. This complicated the isolation process leading to twice as many metabolites as positions hydroxylated. Thus, *Glomerella fusarioides* (ATCC 9552) furnished at least

(1) The Ben May Laboratory for Cancer Research, University of Chicago, Chicago 37, Ill.

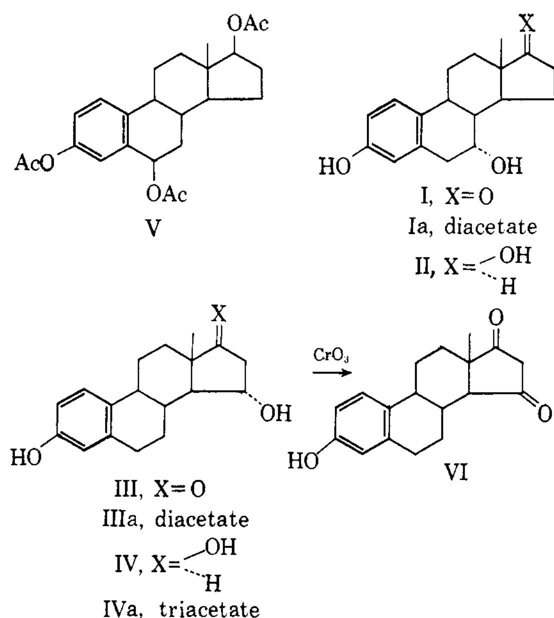
(2) For a most recent review on this subject with citations of earlier review papers, *cf.* C. Tamm, *Angew. Chem.*, **74**, 225 (1962).

(3) K. A. Kita, J. L. Sardinas, and G. M. Shull, *Nature*, **190**, 627 (1961).

(4) B. F. Stimmel, T. E. Bucknell, and V. Notchev, *Federation Proc.*, 115 (1960).

(5) J. Iriarte, H. J. Ringold, and C. Djerassi, *J. Am. Chem. Soc.*, **80**, 6105 (1958).

four products, the known 7α-hydroxyestrone (I)[5] and 7α-hydroxyestradiol (II),[5] as well as two new metabolites shown in the sequel to be 15α-hydroxyestrone (III, m.p. 226–230°, [α]D +197° in ethanol) and 15α-hydroxyestradiol (IV, m.p. 248–250°, [α]D +163° in dioxane). The same products were obtained using *Glomerella glycines* (ATCC 3422) and the morphologically unrelated *Aspergillus carneus* (Centraalbureau voor Schimmelcultures, Baarn, Holland).



15α-Hydroxyestrone was further characterized as the diacetate and 15α-hydroxyestradiol as the triacetate. The location of the newly introduced hydroxyl group became evident upon examination of the spectra of the diketone VI prepared by chromic acid oxidation of either III or IV. Its ultraviolet spectrum in methanolic potassium hydroxide showed an intense peak at 277 mμ (ε 15,000), and the infrared spectrum exhibited peaks at 5.66 and 5.78 μ (in addition to benzenoid absorption) in close harmony with the data reported by Bernstein, *et al.*,[6] for 15-ketoandrostenedione ($\lambda_{max}^{alc\ KOH}$ 277 mμ (ε 19,700), λ_{max}^{KBr} 5.66 and 5.76 μ). Additional and most convincing evidence for the presence of a 1,3-diketone system was derived from the n.m.r. spectrum, which exhibited a singlet representing two protons at τ 7.03, characteristic of a methylene group flanked by two carbonyl groups.[7] The fact that this peak integrates to two protons indicates that VI must be completely in the diketone form, a finding that is supported by the failure of VI to give a coloration with ferric chloride and by the ultraviolet spectrum in neutral medium, which is essentially identical with that of estrone. Having established the position of the newly introduced hydroxyl group, its orientation follows from the strongly positive molecular rotation contribution of that group in both 15-hydroxyestrone (ΔMD +141° in dioxane) and 15-hydroxyestradiol (ΔMD +249° in alcohol).[8] For the 15-ketone VI we propose the C–D *cis*-fused 14β-structure since it is recovered unchanged on treatment with base under conditions that will readily isomerize 15-keto steroids[9] and related *trans*-indanones[10,11] to their *cis* isomers. Moreover, the negative rotation contribution of the 15-keto group in VI (ΔMD −226° in methanol) rather than a positive increment as in 15-ketoprogesterone (ΔMD +53° in chloroform)[9] lends additional support to the *cis* fusion of rings C and D.[12]

The isomerization of the *trans*- to the *cis*-indanone could only have occurred under the slightly acidic conditions of the Jones oxidation. Such a reaction, although not previously encountered under these conditions, is not unanticipated, in view of the fact that Dreiding models indicate a greater strain in the *trans*-fused 15,17-diketone system than is known to exist in the corresponding *trans*-fused monoketones. Relief of that strain by enolization of the 15-keto group towards C-14 would, therefore, be expected to occur more readily in the present case.

The capacity of microorganisms of the genus *Glomerella* to effect hydroxylation of steroidal substrates is not confined to the phenolic estrogens. *Glomerella* species including *G. fusarioides* have been reported to hydroxylate cortexolone and related substrates in the 11α-position.[13] To rationalize this shift in the locus of hydroxylation with the change in substrate, we wish to point to the fact that carbon atoms 15, 14, 13, and 18 occupy the same positions relative to each other as carbon atoms 11, 9, 10, and 19. On the premise then that the presence of the latter arrangement of carbon atoms is instrumental in directing enzymic attack to C-11, its absence in the ring A aromatic steroids might be expected to shift the attack to the terminus of the related sequence, namely, C-15.

Fermentation of estrone with *Mortierella alpina* (ATCC 8979)[14] gave in moderate yield the known 6β-hydroxyestradiol,[15] isolated as the triacetate. It is interesting to note in this connection that 6β-hydroxyestradiol has been isolated by Breuer, *et al.*,[15b] from incubates of estradiol with rat liver microsomes.

7α-Hydroxyestrone and 15α-hydroxyestradiol had, respectively, 1/3000 and 1/1000 the uterotrophic

(6) (a) S. Bernstein, L. I. Feldman, W. S. Allen, R. H. Blank, and C. E. Linden, *Chem. Ind.* (London), 111 (1956); (b) S. Bernstein, M. Heller, L. I. Feldman, W. S. Allen, R. H. Blank, and C. E. Linden, *J. Am. Chem. Soc.*, **82**, 3685 (1960).

(7) The signal for the methylene protons in the analogous cyclopentene-3,5-dione [C. H. DePuy and E. F. Zawesky, *ibid.*, **81**, 4920 (1959)] is at τ 7.10: private communication from Professor DePuy.

(8) The molecular rotation contributions for the hydroxyl group in 15α- and 15β-hydroxyandrostenedione in methanol are ΔMD +107 and −71°, respectively (*cf.* ref. 6). These values are in good agreement with those for the 15-hydroxy derivatives of progesterone [J. Fried, R. W. Thoma, D. Perlman, J. E. Herz, and A. Borman, *Recent Progr. Hormone Res.*, **11**, 157 (1955)] and of cortexolone (ref. 6).

(9) C. Djerassi, L. B. High, J. Fried, and E. F. Sabo, *J. Am. Chem. Soc.*, **77**, 3673 (1955).

(10) J. F. Biellman, D. Francetić, and G. Ourisson, *Tetrahedron Letters*, **18**, 4 (1960).

(11) J. Fried and E. F. Sabo, *J. Am. Chem. Soc.*, **84**, 4356 (1962).

(12) The related 15-ketoandrostenedione described by Bernstein, *et al.* (ref. 6a,b), shows a very similar ΔMD for the 15-keto group (−194°). It is more than likely, therefore, that both compounds possess rings C and D in *cis* fusion. The possibility that an enol form could cause the observed negative rotation contribution as discussed by Bernstein, *et al.* (*cf.* footnote 14 in ref. 6b), is ruled out in our case by the n.m.r. and ultraviolet data.

(13) F. Carvajal, U. S. Patent 2,985,563 (1961).

(14) *Mortierella* species including *M. alpina* have been claimed to effect 6β-hydroxylation of progesterone [L. I. Feldman, N. E. Rigler, A. J. Shay, and B. E. Nielsen, U. S. Patent 3,060,101 (1962)] and 1-hydroxylation of 9α-fluorohydrocortisone [L. I. Feldman, N. E. Rigler, and A. J. Shay, U. S. Patent 2,462,423 (1961)].

(15) (a) O. Wintersteiner and M. Moore, *J. Am. Chem. Soc.*, **81**, 442 (1959); (b) H. Breuer, R. Knuppen, and G. Pangels, *Nature*, **190**, 720 (1961); *Biochim. Biophys. Acta*, **65**, 1 (1962).

activity of estradiol benzoate in the immature female mouse assay.[16]

Experimental

All melting points are uncorrected in capillaries. Rotations are in chloroform unless otherwise specified. Ultraviolet spectra were determined on a Cary 11 spectrometer, infrared spectra on a Perkin-Elmer 21 spectrometer, and n.m.r. spectra on a Varian A-60 spectrometer in deuteriochloroform solution with tetramethylsilane as internal standard. The following paper chromatographic systems were used to determine the progress of the fermentations. The toluene–propylene glycol system of Zaffaroni, *et al.*,[17] was used for determination of residual substrate. For separation of the products, a modification of the B5 system of Bush[18] was used, in which the ratio of benzene–methanol–water was 1:1:1, and the procedure was carried out at room temperature instead of at 30–38°.

Fermentation of Estrone with *Glomerella fusarioides* (ATCC 9552).—Surface growth from each of five 3-week agar slant cultures of *Glomerella fusarioides* (ATCC 9552) (the slant containing nutrient medium A–glucose, 10 g.; Difco yeast extract, 2.5 g.; K_2HPO_4, 1 g.; agar, 20 g.; and distilled water to 1 l.) was suspended in 2.5 ml. of an 0.01% sodium lauryl sulfate aqueous solution. One-milliliter portions of the suspension were used to inoculate ten 250-ml. conical flasks (each containing 50 ml. of sterilized nutrient medium B–dextrose, 10 g.; cornsteep liquor, 6 g.; $NH_4H_2PO_4$, 3 g.; Difco yeast extract, 2.5 g.; $CaCO_3$, 2.5 g.; and distilled water to 1 l.) After 5 days of incubation at 25° with continuous rotary agitation (280 cycles per min., 2-in. radius), 10% (v./v.) transfers were made to one-hundred 250-ml. conical flasks each containing 50 ml. of fresh sterilized medium B. To each flask was added 0.25 ml. of a sterile solution of the steroid in N,N-dimethylformamide (60 mg./ml.) so that the concentration of steroid was 300 μg./ml. After 48 hr. of further incubation, the contents of the flasks were pooled and filtered through a Seitz clarifying pad. The flasks, mycelium, and pad were washed with successive 50-ml. portions of warm water.

The combined filtrate and washings (5300 ml.) were extracted with three 1-l. portions of chloroform, and the combined chloroform extracts were washed with water and evaporated to dryness *in vacuo*. The resulting crude residue (654 mg.) on trituration with ethyl acetate furnished 290 mg. of a residue, which on recrystallization from methanol furnished 153 mg. of 7α-hydroxyestrone (I), m.p. 260–262°; $[\alpha]^{23}D$ +111° (*c* 0.42, 95% ethanol), +117° (dioxane); λ_{max}^{alc} 281 mμ (ϵ 2380); in 2.5% KOH in methanol λ_{max} 297 mμ (ϵ 2980); λ_{max}^{Nujol} 2.92, 5.80, 5.98, 6.15, 6.27, and 6.63 μ; lit.[5] m.p. 260–262°, $[\alpha]D$ +124° in dioxane.

Anal. Calcd. for $C_{18}H_{22}O_3$ (286.36): C, 75.49; H, 7.74. Found: C, 75.71; H, 7.81.

The ethyl acetate mother liquors from which most of the 7α-hydroxyestrone had been removed were evaporated to dryness *in vacuo* and taken up in chloroform; the resulting crystals were removed by filtration. Concentration of the mother liquors produced crystalline material (275 mg.), which on recrystallization from ethyl acetate produced an additional 16 mg. of 7α-hydroxyestrone melting at 257–258°. Evaporation of the ethyl acetate mother liquors to dryness and crystallization of the residue from chloroform–ethyl acetate furnished 15α-hydroxyestrone (III, 73 mg.), which after additional recrystallization from the same solvent mixture had m.p. 228–230°; $[\alpha]^{23}D$ +197° (*c* 0.49, 95% ethanol); λ_{max}^{alc} 281 mμ (ϵ 2290); in 2.5% KOH in methanol λ_{max} 297 mμ (ϵ 3030); λ_{max}^{Nujol} 3.00, 5.78, 6.19, 6.29, and 6.66 μ.

Anal. Calcd. for $C_{18}H_{22}O_3$ (286.36): C, 75.49; H, 7.74. Found: C, 75.38; H, 7.39.

7α-Hydroxyestrone 3,7-Diacetate (Ia).—Fifty milligrams of 7α-hydroxyestrone was acetylated with 0.5 ml. of acetic anhydride in 0.5 ml. of dry pyridine at room temperature for 18 hr. Evaporation of the reagents furnished 65 mg. of crude material, which on recrystallization from ether–hexane gave 44 mg. of pure 7α-hydroxyestrone diacetate (Ia), m.p. 135–137°; $[\alpha]^{23}D$ +51° (*c* 0.46); lit.[5] m.p. 140–141°, $[\alpha]D$ +65° in chloroform.

Anal. Calcd. for $C_{22}H_{26}O_5$: C, 71.33; H, 7.08. Found: C, 71.05; H, 6.92.

The infrared spectrum of this sample was identical with that of an authentic sample of 7α-hydroxyestrone diacetate, kindly supplied by Dr. H. Ringold.

15α-Hydroxyestrone 3,15-Diacetate (IIIa).—Sixty-two milligrams of 15α-hydroxyestrone was acetylated as described above. The crude residue (78 mg.) was recrystallized from ether–hexane and gave 48 mg. of pure 15α-hydroxyestrone diacetate (IIIa), m.p. 142–143°; $[\alpha]^{23}D$ +201° (*c* 0.64); λ_{max}^{Nujol} 5.65, 5.73, 6.20, 6.68, and 7.95 μ.

Anal. Calcd. for $C_{22}H_{26}O_5$ (370.43): C, 71.33; H, 7.08. Found: C, 71.13; H, 7.26.

15α-Hydroxyestradiol (IV).—To a solution of 43 mg. of 15α-hydroxyestrone in 4.5 ml. of methanol was added 45 mg. of sodium borohydride. The reaction mixture was allowed to remain at room temperature for 1 hr., diluted with water, and acidified with glacial acetic acid; the methanol was evaporated *in vacuo*. The resulting suspension was extracted with ethyl acetate; the ethyl acetate extract was washed with water, dried over sodium sulfate, and evaporated to dryness *in vacuo*. The residue on recrystallization from ethyl acetate furnished pure 15α-hydroxyestradiol (IV, 37 mg.), m.p. 248–250°; $[\alpha]^{23}D$ +163° (*c* 0.48, dioxane); λ_{max}^{Nujol} 2.90, 3.00, 3.15, 6.15, 6.30, and 7.65 μ.

Anal. Calcd. for $C_{18}H_{24}O_3$ (288.37): C, 74.97; H, 8.39. Found: C, 74.91; H, 8.26.

15α-Hydroxyestradiol 3,15,17-Triacetate (IVa).—Acetylation of 15α-hydroxyestradiol with pyridine and acetic anhydride as described above gave the crystalline triacetate, which after recrystallization from acetone–hexane had the following properties: m.p. 154–156°; $[\alpha]^{23}D$ +128° (*c* 0.60); λ_{max}^{alc} 257 mμ (ϵ 770), 266 mμ (ϵ 755); λ_{max}^{Nujol} 5.65, 5.76, 6.23, 6.32, 6.70, and 8.02 μ.

Anal. Calcd. for $C_{24}H_{30}O_6$ (414.48): C, 69.54; H, 7.30. Found: C, 69.39; H, 7.32.

15-Keto-14β-estrone (VI).—To a solution of 48 mg. of 15α-hydroxyestrone (IV) in 2 ml. of acetone was added 1.3 ml. of a solution containing 20 mg. of chromium trioxide and 32 mg. of concentrated sulfuric acid per milliliter of 90% aqueous acetone. The mixture was allowed to remain at room temperature for 30 min., following which excess chromium trioxide was reduced by the addition of a few drops of methanol. Chloroform and water were added; the chloroform layer was washed with water, dried over sodium sulfate, and evaporated to dryness *in vacuo*. The residue (35 mg.) was dissolved in 4 ml. of ethyl acetate and 4 ml. of hexane and chromatographed on 2 g. of silica gel. Elution with the same solvent mixture (50 ml.) furnished crystals which after recrystallization from acetone–hexane afforded the 15-keto-14β-estrone (VI), m.p. 193–195°; $[\alpha]^{23}D$ +79° (*c* 0.30), +69° (*c* 0.46, methanol); λ_{max}^{alc} 279 mμ (ϵ 2750), shoulder at 245–250 mμ (ϵ 3100); in 2.5% KOH in methanol λ_{max} 240 mμ (ϵ 8300), 277 mμ (ϵ 15,000); λ_{max}^{KBr} 2.98, 5.66, 5.78, 5.91 (acetone of crystallization), 6.20, 6.31, and 6.64 μ; n.m.r., τ 8.93 (s, 19-Me), 7.81 (s, three protons, acetone), 7.18 (two protons, 14-CH and 9-CH), 7.03 (s, two protons, 16-CH_2), and 3.38, 3.30, and 2.82 (one proton each, aromatic CH).

Anal. Calcd. for $C_{18}H_{20}O_3$ (284.34): C, 76.03; H, 7.09. Found: C, 76.04; H, 7.03.

Fermentation of Estrone with *Glomerella glycines* (ATCC 3422).—Following the fermentation procedure described above but substituting a culture of *Glomerella glycines* (ATCC 3422) for the *G. fusarioides*, supplying 80 flasks of the fermentation medium with 500 μg./ml. of steroid instead of 300 μg./ml., and carrying out the final incubation stage for 24 hr. instead of 48 hr., there was obtained a combined filtrate and washings possessing a volume of 5020 ml. This solution was extracted with three 1.6-l. portions of chloroform, and the combined chloroform extracts were filtered and evaporated to dryness *in vacuo*. The resulting residue (390 mg.) on trituration with ethyl acetate furnished 134 mg. of crystalline material, which on recrystallization from the same solvent gave 107 mg. of pure 7α-hydroxyestrone (I). The ethyl acetate mother liquors from the trituration of the crude residue were taken to dryness and triturated with chloroform. The solid residue which weighed 158 mg. was dissolved in ethyl acetate and chromatographed on a column of 8 g. of neutral alumina. Ethyl acetate (500 ml.) eluted crystalline material which after having been triturated with chloroform and recrystallized from ethyl acetate–chloroform furnished pure 15α-hydroxyestrone (III), m.p. 228–230°, identical in all respectis with the material obtained by fermentation with *Glomerella fusarioides*.

Extended Fermentation of Estrone with *Glomerella glycines* (ATCC 3422).—Following the general procedure described above, but carrying out the final incubation stage for 7 days instead of

(16) The authors appreciate the cooperation of Dr. L. J. Lerner and his associates in performing these assays.

(17) A. Zaffaroni, R. B. Burton, and E. H. Keutman, *Science*, **111**, 6 (1950).

(18) I. E. Bush, *Biochem. J.*, **50**, 370 (1952).

48 hr. in ten 2-l. flasks each containing 500 ml. of medium B supplied with 500 μg. of steroid per milliliter, the combined filtrate and washings had a volume of 6000 ml. This solution was extracted with three 3-l. portions of chloroform and the combined chloroform extracts were filtered and evaporated to dryness *in vacuo*. Fractional crystallization of the resulting residue (706 mg.) from ethyl acetate gave as the most insoluble component 43 mg. of 15α-hydroxyestradiol (IV) melting at 248–250° and identical in all respects with the material described above. This was followed by several fractions melting at about 252–256° which on recrystallization from ethyl acetate gave 85 mg. of 7α-hydroxyestrone (I), m.p. 258–260°.

An additional amount of 15α-hydroxyestradiol was obtained by extraction of the chloroform-extracted broth with two 1-l. portions of methyl isobutyl ketone. Evaporation of these extracts furnished 110 mg. of crystalline material which on crystallization from methanol furnished 55 mg. of 15α-hydroxyestradiol melting at 247–249°.

Fermentation of Estradiol with *Aspergillus carneus* (Baarn).—Surface growth from a 3-week agar slant culture of *Aspergillus carneus* (Baarn), the slant containing nutrient medium A, was suspended in 2.5 ml. of a 0.01% sodium lauryl sulfate aqueous solution. One-milliliter portions of the suspension were used to inoculate two 250-ml. conical flasks, each containing 50 ml. of the sterilized nutrient medium B. After 72 hr. of incubation at 25° with continuous rotary agitation (280 cycles per min., 2-in. radius), 10% (v./v.) transfers were made to twelve 250-ml. conical flasks each containing 50 ml. of fresh sterilized medium B. These were incubated under the conditions described above for 4 days, after which another 10% (v./v.) transfer was made to 100 additional 250-ml. flasks containing 50 ml. of fresh sterilized medium B. The estradiol was supplied by adding to each flask 0.25 ml. of a sterile solution of the steroid in N,N-dimethylformamide (100 mg./ml.) so that the concentration of steroid was 500 μg./ml. After 5 days of further incubation, the contents of the flasks were pooled and filtered through a Seitz clarifying pad. The flasks, mycelium, and pad were washed with successive 50-ml. portions of warm water.

The combined filtrate and washings (4250 ml.) were extracted with three 1-l. portions of methyl isobutyl ketone. The combined extracts were evaporated to dryness leaving 1.57 g. of a semicrystalline residue. This material was triturated with chloroform and the crystalline residue (1.25 g.) was triturated again with acetone. Recrystallization from acetone and finally from 95% ethanol produced pure 15α-hydroxyestradiol (IV), m.p. 246–248°. The combined mother liquors obtained in the above triturations with chloroform and acetone were evaporated to dryness *in vacuo* and treated with ethyl acetate. This produced 425 mg. of crystals consisting mainly of 7α-hydroxyestradiol (II) contaminated by a small amount of 7α-hydroxyestrone (I) as well as a mother liquor, which was concentrated to a volume of 10 ml. for chromatography as described below. To separate the two components, the material was recrystallized from 95% alcohol which furnished 44 mg. of 7α-hydroxyestrone (I) identified by its infrared spectrum. The mother liquors were evaporated to dryness and recrystallized from ethyl acetate furnishing 75 mg. of pure 7α-hydroxyestradiol, m.p. 255–257°, lit.[5] m.p. 254–260°.

Anal. Calcd. for $C_{18}H_{24}O_3$ (288.37): C, 74.97; H, 8.39. Found: C, 75.18; H, 8.39.

The infrared spectrum of this sample was identical with that of an authentic specimen.

The ethyl acetate mother liquor (10 ml.) described above was chromatographed on 32 g. of acid-washed alumina. Elution of the column with ethyl acetate (350 ml.) furnished 210 mg. of crystalline material which after recrystallization from acetone–ether yielded 50 mg. of 7α-hydroxyestrone (I). Continued elution with ethyl acetate–acetone 1:1 (250 ml.) gave an additional 25 mg. of 7α-hydroxyestrone. Subsequent elution with 350 ml. of ethyl acetate–acetone 1:1 and with acetone (300 ml.) furnished 210 mg. of crude crystals which on recrystallization from acetone yielded 28 mg. of pure 15α-hydroxyestradiol (IV), m.p. 248–250°.

6β-Hydroxylation of Estrone by *Mortierella alpina* (ATCC 8979).—Surface growth from a 3-week agar slant culture of *Mortierella alpina* (ATCC 8979), the slant containing nutrient medium A, was suspended in 2.5 ml. of a 0.01% sodium lauryl sulfate aqueous solution. One-milliliter portions of the suspension were used to inoculate two 250-ml. conical flasks, each containing 50 ml. of the sterilized nutrient medium B. After 48 hr. of incubation at 25° with continuous rotary agitation (280 cycles per min., 2-in. radius), 10% (v./v.) transfers were made to twelve 250-ml. conical flasks each containing 50 ml. of fresh sterilized medium B. These were incubated under the conditions described above for 48 hr., after which another 10% (v./v.) transfer was made to 100 additional 250-ml. flasks containing 50 ml. of fresh sterilized medium B. The estrone was supplied by adding to each flask 0.25 ml. of a sterile solution of the steroid in N,N-dimethylformamide (100 mg./ml.) so that the concentration of steroid was 500 μg./ml. After 5 days of further incubation, the contents of the flasks were pooled and filtered through a Seitz clarifying pad. The flasks, mycelium, and pad were washed with successive 50-ml. portions of warm water.

The combined filtrate and washings (6000 ml.) were extracted with three 2-l. portions of methyl isobutyl ketone; the extracts were washed with water and evaporated to dryness *in vacuo*. The residual oily material, 3.5 g., was dissolved in benzene (100 ml.) and chromatographed on 60 g. of neutral alumina. The column was washed successively with 400-ml. portions of benzene–ethyl acetate (1:1) and ethyl acetate, and these eluates were discarded. Subsequent elution with methanol produced 600 mg. of dark brown noncrystalline material which contained the desired 6β-hydroxyestradiol. For further purification it was dissolved in 50 ml. of ethyl acetate and chromatographed on 24 g. of neutral alumina. Elution with ethyl acetate (800 ml.) produced mainly nonpolar material which was discarded. Elution with 200 ml. of ethyl acetate–acetone (3:1), 200 ml. (1:1), 1 l. of acetone, and finally with 200 ml. of methanol furnished amorphous material which consisted essentially of 6β-hydroxyestradiol as shown by paper chromatography. The fractions were combined and acetylated with 2 ml. of dry pyridine and 2 ml. of acetic anhydride. Evaporation of the reagents left a crystalline residue which after several recrystallizations from ether–hexane furnished 40 mg. of pure 6β-hydroxyestradiol triacetate (V), m.p. 173–175°, $[\alpha]^{23}D$ +57° (c 0.45, chloroform); lit.[15] m.p. 176–178°, $[\alpha]D$ +53° in chloroform.

Anal. Calcd. for $C_{24}H_{30}O_6$ (414.48): C, 69.54; H, 7.30. Found: C, 69.61; H, 7.23.

The infrared spectrum of this substance was identical with that of an authentic sample of 6β-hydroxyestradiol triacetate kindly furnished by Dr. O. P. Wintersteiner.

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