of silica gel and allowed to remain on the adsorbent for 2 days. Elution with ether–petroleum ether mixtures yielded 187 mg. of crude product which after successive recrystallizations from acetone–petroleum ether yielded 102 mg. of pure 3β,17β-diacetoxyallopregnane-20-one (V), m.p. 226–229°, reported 227–229°[7]; the infrared spectrum was identical with that of an authentic sample.[8]

Twenty-seven mg. of IV was heated to 235° for 10 minutes. The product solidified upon cooling and was recrystallized from acetone–petroleum ether to yield 22 mg. of 3β,17β-diacetoxyallopregnane-20-one (V) identical in infrared spectrum and melting point with the known compound.

Allopregnane-3β,17α,20α-triol 3,20-Diacetate (VII) from 17α,20β-Epoxyallopregnane-3β,20α-diol Diacetate (IV).—Approximately 100 mg. of lithium aluminum hydride was added to a solution of 58 mg. of IV in 1 ml. of benzene and 9 ml. of ethyl ether. The solution was stirred for 1 hour and then was heated to boiling for 5 minutes. Ethyl acetate was added followed by dilute hydrochloric acid. The organic phase was washed successively with water and dilute sodium hydroxide, again with water and dried over sodium sulfate. After removal of the solvent, 47 mg. of product was obtained and this was acetylated with acetic anhydride and pyridine at room temperature for 16 hours. The acetate was isolated in the usual manner and was recrystallized from petroleum ether containing a trace of acetone. Two crops were obtained: the first, 26 mg., m.p. 247–250°; the second, 10 mg., m.p. 240–245°. Chromatography of the mother liquors upon silica gel followed by recrystallization yielded 8 mg., m.p. 240–245°. All the products exhibited an infrared spectrum indistinguishable from that of an authentic sample of allopregnane-3β,17α,20α-triol 3,20-diacetate (diacetate of Reichstein's "Substance O").

(7) C. W. Shoppee and D. Prins, *Helv. Chim. Acta*, **26**, 185 (1943).

(8) We are indebted to Dr. R. B. Turner of Rice Institute, Houston, Texas, for this reference compound.

3α,21-Diacetoxy-17α,20β-epoxypregnane-11-one (VIII).—A solution of 65 mg. (0.156 mm.) of 3α,21-diacetoxy-Δ^{17}-pregnene-11-one[9] in 1 ml. of benzene was mixed with 1 ml. of a 0.398 *M* benzene solution of perbenzoic acid (0.785 mm.). After 4 days at room temperature the solution was diluted with ether, washed with dilute sodium carbonate solution and water, and was dried over sodium sulfate. After removal of the solvent and crystallization from petroleum ether, 42 mg., m.p. 156–165°, together with a second crop of 11 mg., m.p. 150–162°, was obtained. Recrystallization from petroleum ether yielded 3α,21-diacetoxy-17α,20β-epoxypregnane-11-one (VIII) as needles, m.p. 168–170°, $[\alpha]^{26}D$ +77.6° (chloroform).

Anal. Calcd. for $C_{25}H_{36}O_6$: C, 69.42; H, 8.39. Found: C, 69.44; H, 8.41.

17α-Hydroxy-3α,20α,21-triacetoxypregnane-11-one (IX) from VIII.—A solution of 32 mg. of 3α,21-diacetoxy-17α,-20β-epoxypregnane-11-one (VIII) in 11 ml. of redistilled glacial acetic acid was heated under reflux for 24 hours. The solvent was removed under diminished pressure and the residue oil was chromatographed upon a partition column in which silica gel acted as the support for 95% ethanol. Solution was achieved with 1:1 methylene chloride–petroleum ether containing 1% ethanol. The product was recrystallized and 11 mg. of crude product was obtained. Recrystallization yielded 4 mg. of m.p. 211–214°, 3 mg., m.p. 208–213°, and 2 mg., 201–209°; the infrared spectra of these products was indistinguishable from an authentic sample of 3α,20α,21-triacetoxy-17α-hydroxypregnane-11-one, m.p. 213–214°.[9,10]

(9) We wish to express our gratitude to Dr. L. H. Sarett, Merck & Co., Inc., Rahway, New Jersey, for his generosity in supplying us with this compound.

(10) L. H. Sarett, THIS JOURNAL, **71**, 1169 (1949).

NEW YORK, N. Y.

[FROM THE SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH]

Studies of Steroid Ring D Epoxides of Enol Acetates; A New Synthesis of Estriol and of Androstane-3β,16α,17β-triol[1]

BY NORMA S. LEEDS, DAVID K. FUKUSHIMA AND T. F. GALLAGHER

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A generally applicable method for the preparation of steroid ring D glycols in good yield and with a high degree of stereospecificity has been developed. The procedure consists in the formation of an enol acetate of a 17-ketosteroid followed by epoxidation with perbenzoic acid. The oxide ring is attached to C-16 and C-17 in the α-configuration. With or without prior acid hydrolysis, the epoxyacetate is reduced with lithium aluminum hydride and the principal product obtained is the 16α,17β-glycol. The method is illustrated by the preparation of estriol from estrone enol acetate in 66% yield without isolation of any intermediates, and by the preparation of androstane-3β,16α,17β-triol from isoandrosterone. By alkaline hydrolysis of the intermediary ketol acetate the procedure can be altered to afford an equally successful synthesis of 16β,17β-ring D glycols. The rearrangement of ring D epoxyacetates by chromatography or heating above the melting point is shown to proceed with retention of configuration at C-16.

Because of the interest in steroid ring D ketols as metabolites of hormones and potentially useful intermediates in the synthesis of ring D glycols, it was desired to provide a generally applicable and simple synthesis for these compounds. In particular, an efficient synthesis of estriol, one of the metabolites of the estrogenic hormone estradiol was sought. It seemed reasonable to anticipate that epoxidation of the enol ester of estrone would proceed as with the C-20 ketosteroids[2] and would furnish a key intermediate, X, that could be readily reduced to estriol. This, indeed, proved to be the fact. Two other essentially identical procedures for the partial synthesis of estriol from estrone have been reported.[3,4] The present method has the advantage of a higher yield and greater simplicity in that the whole operation can be accomplished without isolation of any intermediates.

For reasons that will be clear, it was preferable to carry out the initial studies on a neutral compound and 3β-hydroxyandrostane-17-one (isoandrosterone, (I)) was chosen as the starting material. Upon treatment with isopropenyl acetate, in the presence of catalytic amounts of sulfuric acid, the enol acetate II was obtained in better than 80% yield. With dilute perbenzoic acid in benzene at room temperature, II was readily converted to 16α,-17α-epoxyandrostane-3β,17β-diol diacetate (III).

(1) This investigation was supported by grants from the Anna Fuller Fund, the Lillia Babbitt Hyde Foundation, the Teagle Foundation, and the National Cancer Institute, United States Public Health Service (C-440).

(2) T. H. Kritchevsky and T. F. Gallagher, *J. Biol. Chem.*, **179**, 507 (1949); THIS JOURNAL, **73**, 184 (1951).

(3) M. N. Huffman and W. R. Miller, *Science*, **100**, 312 (1944); M. N. Huffman, *J. Biol. Chem.*, **169**, 167 (1947).

(4) A. Butenandt and E. L. Schäffler, *Z. Naturforsch.*, **1**, 82 (1946).

When the epoxide III was chromatographed upon silica gel, a rearrangement of the product occurred with the opening of the epoxide ring and migration of the 17-acetoxyl group to yield 3β,16α-diacetoxyandrostane-17-one (V). The same product was obtained when III was heated above its melting point or subjected to acid hydrolysis followed by reacetylation. Reduction of either III or V by means of lithium aluminum hydride yielded androstane-3β,16α,17β-triol (IVa), isolated and characterized as both the free triol IVa and the triacetate IVb. The latter proved to be identical with the product obtained from the catalytic reduction of Δ⁵-androstene-3β,16α,17β-triol triacetate.[5,6] Although this reaction sequence completed the model study for the synthesis of estriol, the structure of the products and intermediates must be discussed in order to clarify the points of similarity and difference between these reactions and those described in the preceding report.[7]

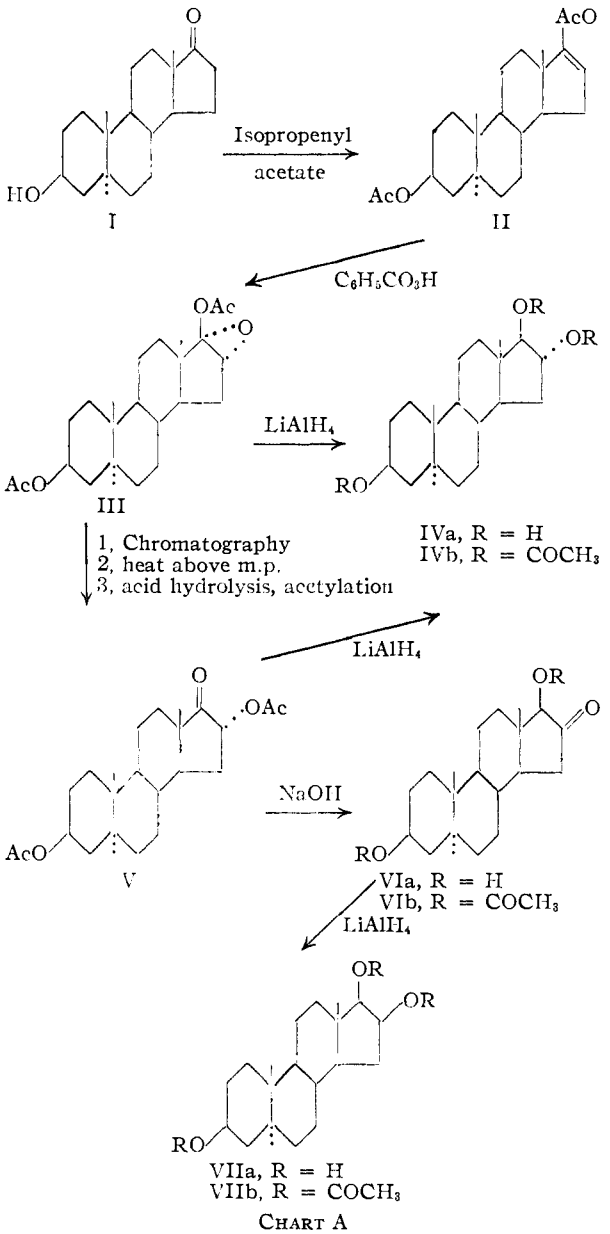

CHART A

When the ketol diacetate V was subjected to alkaline hydrolysis at room temperature, a ketol rearrangement to the more stable 3β,17β-dihydroxyandrostane-16-one (VIa) occurred. This product was identical with the compound prepared by Huffman and Lott[8] from 3β-hydroxy-16-oximinoandrostane-17-one. The ketol rearrangement was predictable from the results obtained by Huffman and Lott[9] in a series of steroid ring D ketols; in all instances, the 17β-hydroxy-16-keto derivative was the sole product obtained under reaction conditions which permitted enolization. Reduction of the ketol acetate VIb by means of lithium aluminum hydride afforded a triol which proved to be identical with androstane-3β,16β,17β-triol (VIIa),[9] the reduction product obtained by catalytic hydrogenation of Δ⁵-androstene-3β,16β,17β-triol. The latter compound is one of the products obtained by Huffman and Lott[9] upon reduction of 16-keto-Δ⁵-androstene-3β,17β-diol with sodium amalgam in dilute ethanol acetic acid. Thus, the configuration of both C-16 and C-17 in VII is well-established and the reduction of a 16-ketone to a 16β-hydroxyl group with lithium aluminum hydride is in agreement with the prediction based upon the "rule of the rear."

The structure of the epimeric triol IV, obtained in this investigation, has been rigorously proved by Hirschmann.[10] Since the α-orientation of the C-16 hydroxyl is certain and since androstane-3β,16α,17β-triol (IV) was obtained from both 16α,17α-epoxyandrostane-3β,17β-diol diacetate (III) and 3β,16α-diacetoxyandrostane-17-one (V), it follows that the oxide ring in III and the ring D acetoxyl group in V were attached to C-16 in the α-configuration. The only alternative to this conclusion would be to presume that III was the β-epoxide and reduction with lithium aluminum hydride was accomplished with inversion not only of the epoxide at C-16, but of the acetoxy group at C-17 as well. Since this seems unlikely, the epoxide ring in III is formulated in the α-orientation.

Therefore, unlike the side chain epoxyacetate, 17α,20β-epoxyallopregnane-3β,20α-diol diacetate reported in the preceding communication[7] where the acetoxyl group was shown to migrate with inversion of configuration, the rearrangement of III to V has been achieved with retention of configuration at C-16. A plausible mechanism whereby the two results can be brought into agreement was suggested to us by Professor Robert B. Woodward of Harvard University in a discussion at the Steroid Conference in New Hampton, New Hampshire. If it be presumed that the acetoxyl group in the ring D epoxyacetate III migrated with inversion, as recorded for the side chain epoxyacetates, the retention of configuration can be ex-

(5) G. F. Marrian and G. C. Butler, *Biochem. J.*, **38**, 322 (1944).
(6) H. Hirschmann, *J. Biol. Chem.*, **150**, 363 (1943).
(7) A. H. Soloway, W. J. Considine, D. K. Fukushima and T. F. Gallagher, THIS JOURNAL, **76**, 2941 (1954).
(8) M. N. Huffman and M. H. Lott, *ibid.*, **73**, 878 (1951).
(9) M. N. Huffman and M. H. Lott, *ibid.*, **71**, 719 (1949). It should be noted that the orientation of the ring D hydroxyl groups reported in this paper is the reverse of that currently accepted.
(10) H. Hirschmann, F. B. Hirschmann and J. W. Corcoran, *Federation Proc.*, **12**, 218 (1953).

plained on the assumption that the 16β-orientation of a hydroxyl or an acetoxy group is unfavored and will rearrange to the more stable 16α-configuration under mild enolizing conditions. We are grateful to Dr. Woodward for his very logical interpretation of this reaction.

Estriol (XII) was obtained in very satisfactory yield by direct reduction of the epoxyacetate X with lithium aluminum hydride or after acid rearrangement to the ketol acetate XI followed by reduction with the same reducing reagent. The simple and efficient procedure described makes estriol a readily

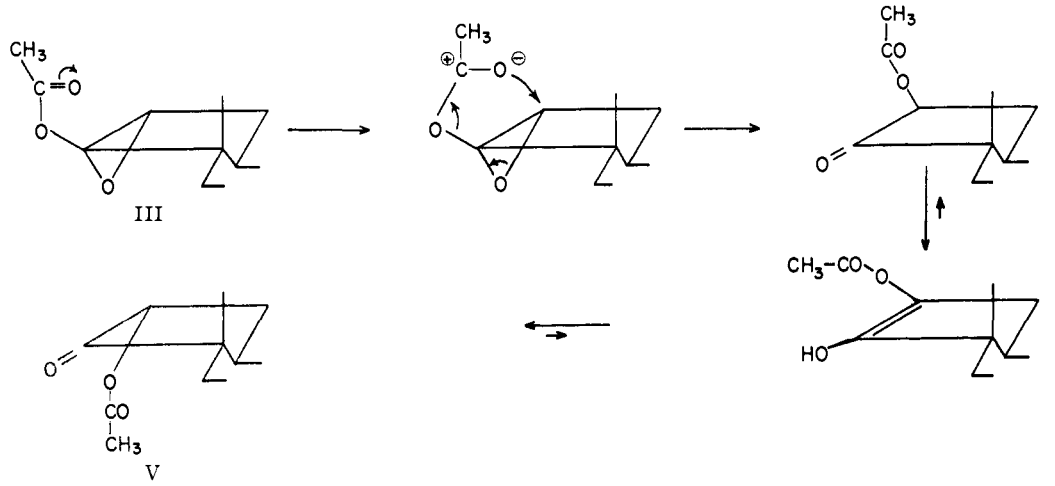

CHART B

An alternative mechanism for the acetoxyl migration without inversion can be described by the following formulation

accessible compound and should serve as a stimulus to further investigation of this biochemically interesting metabolite.

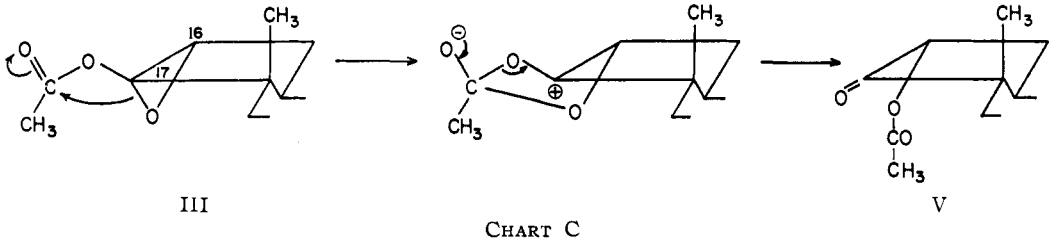

CHART C

It is apparent that further investigation of these alternatives is required and we hope to provide information on these problems at some future time.

With these findings, the synthesis of estriol (XII) from estrone (VIII) proved relatively simple. The enol acetate (IX) of estrone, prepared with isopropenyl acetate, was epoxidized with perbenzoic acid yielding the epoxyacetate (X) which could be readily rearranged to 3,16-α-diacetoxy-$\Delta^{1,3,5}$-estratriene-17-one (XI). The structures of the epoxy acetate and ketol acetate are inferred from the method of preparation and from molecular rotation differences (*cf.* Table I).

Experimental[11]

Δ^{16}-Androstene-3β,17-diol Diacetate (II).[12]—To a solution containing 2.79 g. of isoandrosterone (I) in 20 ml. of isopropenyl acetate was added 1 ml. of catalyst solution (5 ml. of isopropenyl acetate and 0.1 ml. of concentrated sulfuric acid). Approximately 5 ml. of the solvent was distilled over a period of 2 hours. An additional 20 ml. of isopropenyl acetate containing 1 ml. of catalyst solution was added and the solvent of the reaction mixture was concentrated to one-half of its volume by slow distillation over another 2 hours. The solution was then cooled and diluted with ether. The ether solution was washed with cold so-

TABLE I

MOLECULAR ROTATION DIFFERENCES OF 16,17-SUBSTITUTED STEROIDS

		$[\alpha]_D$ ($CHCl_3$)	M_D	ΔM_D (A → B)	ΔM_D (A → C)	ΔM_D (A → D)
A.	Δ^{16}-Androstene-3β,17β-diol diacetate (II)	+ 16.4	+ 61			
B.	16α,17α-Epoxyandrostane-3β,17β-diol diacetate (III)	+ 22.9	+ 89	+27		
C.	3β,16α-Diacetoxyandrostane-17-one (V)	+ 57.1	+223		+162	
D.	3β,17β-Diacetoxyandrostane-16-one (VIb)	−120	−467			−528
A.	$\Delta^{1,3,5,16}$-Estratetraene-3,17-diol diacetate (IX)	+ 90.9	+322			
B.	16α,17α-Epoxy-$\Delta^{1,3,5}$-estratriene-3,17β-diol diacetate (X)	+ 94.9	+352	+30		
C.	3,16α-Diacetoxy-$\Delta^{1,3,5}$-estratriene-17-one (XI)	+122	+451		+129	

(11) All m.p.'s are corrected.

(12) This compound was first prepared in these laboratories by Dr. Hubert Vanderhaeghe.

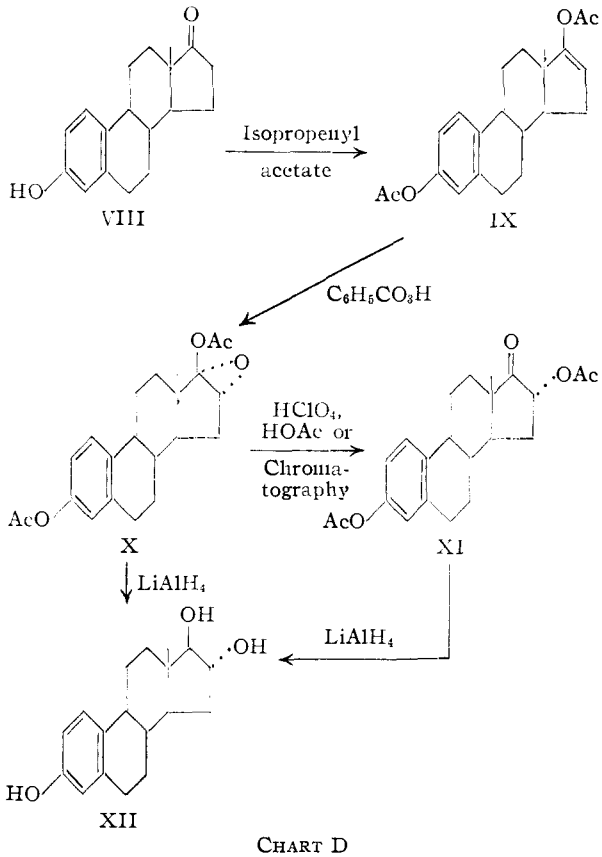

CHART D

dium carbonate solution and with water, dried and the solvent was evaporated. The residue was dissolved in 1 liter of petroleum ether (b.p. 60°) and filtered through 2 g. of alumina. Upon concentration of the filtrate and allowing the solution to stand, 2.67 g. of Δ^{16}-androstene-3β,17-diol diacetate (II), m.p. 169–171°, was obtained. The analytical sample melted at 170–172°, $[\alpha]^{20}D$ +16.4° ($CHCl_3$).

Anal. Calcd. for $C_{23}H_{34}O_4$: C, 73.76; H, 9.15. Found: C, 73.64; H, 9.05.

16α,17α-Epoxyandrostane-3β,17β-diol Diacetate (III).—A solution of 4.41 g. of Δ^{16}-androstene-3β,17-diol diacetate (II) in 386 ml. of 0.05 *M* perbenzoic acid in benzene was allowed to stand for 20 hours at room temperature. The solution was diluted with ether and washed with a solution of sodium hydroxide containing ice and then with water. After drying the organic layer with sodium sulfate and removal of the solvent, 4.94 g. of crude oxidation product was obtained. Recrystallization from acetone–petroleum ether gave 2.03 g. of 16α,17α-epoxyandrostane-3β,17β-diol diacetate (III), m.p. 149–150°. The mother liquor yielded 1.51 g. of oxidation product, m.p. 135–146°, which on further recrystallization gave 626 mg. of epoxide, m.p. 147–149°. The analytical sample melted at 151–152°, $[\alpha]^{23}D$ +22.9° ($CHCl_3$).

Anal. Calcd. for $C_{23}H_{34}O_5$: C, 70.74; H, 8.77. Found: C, 70.56; H, 8.66.

3β,16α-Diacetoxyandrostane-17-one (V). A. By Chromatography.—Following the above procedure, 1.87 g. of Δ^{16}-androstene-3β,17-diol diacetate was treated with 100 ml. of 0.1 *M* perbenzoic acid solution for 2.5 hours. The crude oxidation product (1.96 g.) was acetylated with acetic anhydride and pyridine at room temperature giving 2.32 g. of product which was chromatographed on 400 g. of silica gel. Elution with increasing concentration of ether in benzene yielded crystalline material (1.51 g.) differing in infrared spectrum from the starting material or the pure epoxyacetate III. Repeated recrystallization from methanol gave the following products: 817 mg., m.p. 180–185° (essentially pure V), and the following crude mixtures of V with unknown substance or substances—75 mg., m.p. 170–177°; 108 mg., m.p. 160–165°, and 232 mg., m.p. 140–155°. The analytical sample of 3β,16α-diacetoxyandrostane-17-one (V) melted at 184–185°, $[\alpha]^{23}D$ +57.1° ($CHCl_3$).

Anal. Calcd. for $C_{23}H_{34}O_5$: C, 70.74; H, 8.77. Found: C, 70.87; H, 8.73.

Rearrangement of 16α,17α-epoxyandrostane-3β,17β-diol diacetate (III) to the ketol diacetate V occurred when the *pure* epoxide (401 mg., m.p. 149–150°) was chromatographed on silica gel. Elution with increasing concentration of ether–benzene and recrystallization of the eluates gave 151 mg. of 3β,16α-diacetoxyandrostane-17-one, m.p. 183–185°, identical in infrared spectrum with the pure product prepared by independent means.

B. By Acid Hydrolysis.—To a solution of 173 mg. of 16α,17α-epoxyandrostane-3β,17β-diol diacetate (III) in 20 ml. of methanol was added 5 ml. of 6 *N* aqueous sulfuric acid and the solution was stored at room temperature for 5 days. After dilution with ethyl acetate and washing with cold sodium hydroxide solution and water, the organic phase was dried and the solvent was evaporated. The residue (133 mg.) was acetylated with acetic anhydride and pyridine at room temperature. Recrystallization from acetone–petroleum ether afforded 104 mg. of 3β,16α-diacetoxyandrostane-17-one (V), m.p. 184–185°. Examination of the infrared spectrum of the mother liquor showed that the ketol diacetate V (λ_{max} 1142, 1131, 1108 and 1012 cm.$^{-1}$)[13] was present; there was no indication of the presence of the epoxide III (λ_{max} 1187, 1157, 1149 and 1104 cm.$^{-1}$)[13] or the more stable ketol diacetate, 3β,17β-diacetoxyandrostane-16-one (VIb), (λ_{max} 1136 and 1120 cm.$^{-1}$).[13]

When other samples of III were hydrolyzed in the manner described, the infrared spectrum of the product indicated that the acetoxy groups had been completely removed.

C. By Heating above m.p.—Fifteen mg. of the epoxydiacetate III was immersed in an oil-bath at 200° for 10 minutes. The compound turned amber and attempts to crystallize the product were unsuccessful. Infrared spectral analysis showed the presence of the rearrangement product, 3β,16α-diacetoxyandrostane-17-one (V) as evidenced by the strong absorption at 1142, 1131, 1108 and 1012 cm.$^{-1}$. The characteristic bands (1187, 1157, 1149, 1104 cm.$^{-1}$) of the starting epoxydiacetate used in the identification of this compound in a mixture were not present.

3β,17β-Dihydroxyandrostane-16-one (VIa) and Diacetate (VIb).—A solution of 50 mg. of 3β,16α-diacetoxyandrostane-17-one (V) in 25 ml. of 0.04 *N* sodium hydroxide in 60% aqueous methanol was stored at room temperature for 4 hours. The solution was diluted with ethyl acetate and washed with water, dried and the solvent was removed to give 31 mg. of product. Recrystallization from acetone–petroleum ether and from acetone gave 3β,17β-dihydroxyandrostane-16-one (VIa), m.p. 214–220° dec., $[\alpha]^{23}D$ −149° (dioxan); reported[8] m.p. 217–218° dec. The m.p. of an admixture with a known sample[14] of 3β,17β-dihydroxyandrostane-16-one monohydrate showed no depression and the infrared spectrum of the two samples was identical in all respects.

Anal. Calcd. for $C_{19}H_{30}O_3 \cdot H_2O$: C, 70.31; H, 9.93. Found: C, 70.54; H, 9.60.

Acetylation in the usual way with acetic anhydride and pyridine yielded 3β,17β-diacetoxyandrostane-16-one, m.p. 179–181°, $[\alpha]^{22}D$ −120° ($CHCl_3$). The infrared spectrum was identical with that of the diacetate prepared from the ketol VIa obtained from Dr. M. N. Huffman.

Anal. Calcd. for $C_{23}H_{34}O_5$: C, 70.74; H, 8.77. Found: C, 70.83; H, 8.42.

Androstane-3β,16α,17β-triol (IVa) and Triacetate (IVb). A. From V.—To a solution of 83 mg. of 3β,16α-diacetoxyandrostane-17-one (V) in 10 ml. of ether was added 10 ml. of an ether solution containing 6.2 mmoles of lithium aluminum hydride. The solution was refluxed for one hour, cooled and moist ether and dilute acid were added. The organic material was extracted with ethyl acetate, washed with sodium carbonate and with water and dried over sodium sulfate. Evaporation of the solvent gave 70 mg. of crude triol. Acetylation in the usual manner with acetic anhydride and pyridine at room temperature afforded 85 mg. of crude triacetate. Upon recrystallization from

(13) Only the absorption bands employed for the identification of the compound in the presence of its isomers are reported here.

(14) We are indebted to Dr. M. N. Huffman for his kindness in supplying this authentic product.

acetone–petroleum ether, 59 mg. of androstane-3β,16α,17β-triol triacetate (IVb), m.p. 174–176°, $[\alpha]^{22}D$ −57° ($CHCl_3$) was obtained.

Anal. Calcd. for $C_{25}H_{38}O_6$: C, 69.09; H, 8.82. Found: C, 69.29; H, 8.74.

There was no depression of the m.p. when admixed with an authentic sample[15] of androstane-3β,16α,17β-triol triacetate, m.p. 174.5–176°. The infrared spectrum of the two samples was identical in all regions. Chromatography of the mother liquors on silica gel gave an additional 10 mg. of the triacetate IVb and 14 mg. of a mixture containing IVb together with an unknown compound.

In a separate run a sample of androstane-3β,16α,17β-triol was obtained, m.p. 260–262°, $[\alpha]^{31}D$ −1.9° (ethanol); reported[5] m.p. 257.5–259°, $[\alpha]^{25}D$ 0° (methanol).

Anal. Calcd. for $C_{19}H_{32}O_3$: C, 73.98; H, 10.46. Found: C, 74.07; H, 10.61.

B. From III.—By the above procedure 537 mg. of 16α,17α-epoxyandrostane-3β,17β-diol diacetate (III) was reduced with 18.3 mmoles of lithium aluminum hydride to give 415 mg. of crude product. Acetylation in the usual manner with acetic anhydride and pyridine at room temperature yielded 582 mg. which upon recrystallization from petroleum ether afforded 302 mg. of androstane-3β,16α,17β-triol triacetate (IVb), m.p. 172.5–175°. Chromatography of the mother liquors yielded an additional 34 mg. of IVb, m.p. 174–176° and 69 mg., m.p. 146–160°.

Androstane-3β,16β,17β-triol (VIIa) and Triacetate (VIIb).—An ether solution of 127 mg. of 3β,17β-diacetoxyandrostane-16-one (VIb) was reduced with excess lithium aluminum hydride to yield 106 mg. of product. Upon recrystallization from acetone–methanol, 50 mg. of androstane-3β,16β,17β-triol, m.p. 251–255°, was obtained. The analytical sample melted at 253–256°, $[\alpha]^{23}D$ +13° (ethanol); reported[9] m.p. 251–253°, $[\alpha]^{26}D$ +18° (ethanol).

Anal. Calcd. for $C_{19}H_{32}O_3$: C, 73.98; H, 10.46. Found: C, 73.68; H, 10.52.

The m.p. of the mixture with an authentic sample of androstane-3β,16β,17β-triol, kindly supplied to us by Dr. M. N. Huffmann, was not depressed. The mother liquors were acetylated in the usual manner with pyridine and acetic anhydride at room temperature. Recrystallization from methanol gave 14 mg. of androstane-3β,16β,17β-triol triacetate (VIIb), m.p. 194–196°, $[\alpha]^{27}D$ +22° ($CHCl_3$). The infrared spectrum and m.p. were identical with that of androstane-3β,16β,17β-triol triacetate prepared from an authentic sample of VIIa.

Anal. Calcd. for $C_{25}H_{38}O_6$: C, 69.09; H, 8.82. Found: C, 69.10; H, 8.82.

$\Delta^{1,3,5,16}$-Estratetraene-3,17-diol Diacetate (IX).—A solution of 5.0 g. of estrone (VIII) in 35 ml. of isopropenyl acetate and 1.5 ml. of catalyst solution was refluxed for 2 hours. Approximately 10 ml. of solvent was slowly distilled over a period of an hour. An additional 20 ml. of isopropenyl acetate and 1 ml. of catalyst solution were added and the solution was concentrated to one-half the volume by slow distillation for one hour. The solution was chilled and ether was added. The ether solution was washed with ice-cold sodium bicarbonate solution and with water, dried and the solvent was evaporated. The residue was dissolved in 1 liter of petroleum ether and poured through a short column of alumina which was then washed with an additional 1 liter of petroleum ether. When the solution was concentrated and allowed to crystallize, 5.0 g. of $\Delta^{1,3,5,16}$-estratetraene-3,17-diol diacetate (IX), m.p. 145–149°, was obtained. From the mother liquor an additional 0.7 g. of the enol acetate was obtained. The analytical sample melted at 149–150°, $[\alpha]^{32}D$ +90.9° ($CHCl_3$).

Anal. Calcd. for $C_{22}H_{26}O_4$: C, 74.55; H, 7.39. Found: C, 74.79; H, 7.42.

16α,17α-Epoxy-$\Delta^{1,3,5}$-estratriene-3,17β-diol Diacetate (X).—This compound was prepared from the corresponding enol acetate IX, m.p. 147–150°, by treatment with excess perbenzoic acid in benzene solution at room temperature for 19 hours. After washing the solution with ice-cold sodium hydroxide and sodium chloride solutions, the solvent was evaporated. The product was recrystallized from acetone–petroleum ether to give 16α,17α-epoxy-$\Delta^{1,3,5}$-estratriene-3,17β-diol diacetate, m.p. 150–152°, $[\alpha]^{27}D$ +94.9° ($CHCl_3$); the admixture with the enol acetate IX showed a depression in the m.p.

Anal. Calcd. for $C_{22}H_{26}O_5$: C, 71.33; H, 7.07. Found: C, 71.46; H, 6.96.

3,16α-Diacetoxy-$\Delta^{1,3,5}$-estratriene-17-one (XI).—Upon rearrangement of the epoxydiacetate X on a silica gel column or by perchloric acid in acetic acid, 3,16α-diacetoxy-$\Delta^{1,3,5}$-estratriene-17-one, m.p. 179–180°, $[\alpha]^{28}D$ +122° ($CHCl_3$), was obtained.

Anal. Calcd. for $C_{22}H_{26}O_5$: C, 71.33; H, 7.07. Found: C, 71.19; H, 7.40.

Estriol (XII). A. By Rearrangement of the Epoxide X before Reduction.—Without further purification, 5.0 g. of enol diacetate IX of m.p. 145–149° was dissolved in 25 ml. of benzene and allowed to react for 15 hours with 50 ml. of 0.72 *M* perbenzoic acid. The crude epoxide (5.5 g.) was dissolved in 35 ml. of cold acetic acid and a cold solution of 5 ml. of acetic acid containing 1 ml. of 70% perchloric acid was added. The reaction mixture was allowed to stand for one-half hour at room temperature. Ether was then added and the ether solution was washed with cold sodium carbonate solution and with sodium chloride solution. After drying, the solvent was removed under diminished pressure and the residue was dissolved in 125 ml. of dry ether and 35 ml. of dry benzene. Four grams of lithium aluminum hydride in 700 ml. of dry ether was added dropwise to the solution and after the addition was complete, the reaction mixture was heated under reflux for one hour. Excess reagent was destroyed with ethyl acetate and dilute acid. The reduction product was extracted with large volumes of ethyl acetate and the organic phase was washed with sodium bicarbonate and sodium chloride solutions. After drying and evaporating the solvent, 3.82 g. of product was obtained.

A portion (186 mg.) of the crude estriol was purified by countercurrent distribution using the solvent system cyclohexane–ethyl acetate (1:1) and ethanol–water (1:1) devised by L. L. Engel.[16] The distribution is shown in Fig. 1.

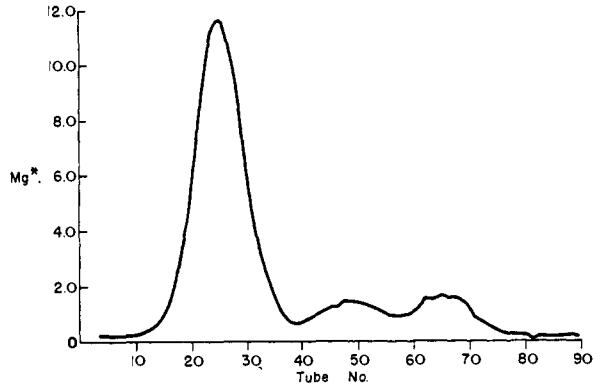

Fig. 1.—Countercurrent distribution of 186 mg. of crude estriol in: upper layer, cyclohexane–ethyl acetate (1:1); lower layer, ethanol–water (1:1); *, based on ϵ_{2800} = 2050.

The combined material in tubes 15–37 had the same distribution coefficient as estriol and very closely approximated a theoretical distribution. There was 130 mg. of estriol on the basis of ultraviolet absorption spectrum and 131 mg. by weight, m.p. 271–275°. A recrystallization of the combined fractions of estriol from methanol–ethyl acetate yielded 87 mg. of estriol, m.p. 273–277°, ϵ_{2800} = 2010 (methanol). The purity of the estriol is evidenced by the countercurrent distribution curve and its m.p. The combined fraction represents 70% of the crude estriol fraction as pure estriol or an over-all yield from the estrone enol diacetate of 66%.

Tubes 40–55 contained 19.4 mg. (11% of the crude estriol fraction) of phenolic substance based on ultraviolet spectra using ϵ_{2800} = 2050 (estriol). The product was not studied further.

(15) We wish to express our appreciation to Dr. H. Hirschmann for determining the mixture melting point as well as supplying us with a sample of IVb for comparison of the infrared spectrum.

(16) L. L. Engel, *Recent Progress Hormone Res.*, **5**, 335 (1950).

Tubes 59–75 contained 19.8 mg. (11% of the crude estriol fraction) of estradiol-17β based on ultraviolet absorption spectrum. This product undoubtedly arose from the lithium aluminum hydride reduction of either estrone acetate, which contaminated the once recrystallized estrone enol diacetate employed in the reactions, or derived from the enol diacetate which was not oxidized with perbenzoic acid.

B. By Reduction of the Epoxide X.—Estrone enol diacetate ($\Delta^{1,3,5,16}$-estratetraene-3,17-diol diacetate (IX)) prepared from 5.0 g. of estrone was poured through a short alumina column and recrystallized four times from petroleum ether. The enol diacetate thus prepared (3.4 g., m.p. 145–149°) was oxidized at room temperature overnight with 3.4 g. of perbenzoic acid in 80 ml. of benzene to give 3.2 g. of epoxyacetate (16α,17α-epoxy-$\Delta^{1,3,5}$-estratriene-3,17β-diol diacetate (X)). The crude epoxide acetate was directly reduced with 3 g. of lithium aluminum hydride in benzene and ether to give 2.40 g. of crude estriol.

A portion (200 mg.) of the crude estriol was purified by countercurrent distribution as above. The individual tubes containing estriol were combined and 124 mg. of product, m.p. 278.5–284°, ϵ_{2800} = 2090 (ethanol), was obtained. Estriol thus obtained represents 62% of the crude estriol fraction or an over-all yield from estrone enol acetate of 54%.

There was 57 mg. of the unknown compound which had a distribution coefficient intermediate between estriol and estradiol. Only 10 mg. of estradiol was obtained, indicating that the starting material, estrone enol diacetate, was relatively free of estrone acetate.

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Testicular Hyaluronidase in Relation to Micelle Formation by Inactivating Agents[1]

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The inactivation of testicular hyaluronidase by an homologous series of sulfated aliphatic alcohols has been studied. The concentrations at which the detergents produced equal rates of inactivation was determined and found to correlate well with the critical micelle concentrations of the detergents. The inactivating effect of the detergents, which increased with time, was not reversed by dilution or precipitating agents. This behavior was not found for polyanionic compounds like heparin, which has been presumed to inhibit competitively on the basis of structural similarity to the substrate. The action of other compounds (*d*-α-tocopheryl phosphate and hexylresorcinol) was found to resemble that of the detergents, and may be similarly interpreted on the basis of the micelle-forming properties possessed by molecules with a strongly hydrophilic group and a long hydrocarbon chain. It is proposed that either anionic micelles interact with hyaluronidase or that a similar micellar aggregate forms at the enzyme surface. A high affinity for the enzyme might be predicted for such an aggregate since it resembles the natural substrates with respect to size, shape and high negative charge. In accordance with this view is the observation that the known organic inhibitors of hyaluronidase which are effective at very low concentration are all negatively charged in solution and either are of high molecular weight or else are very likely associative colloids.

Introduction

The *in vitro* activity of testicular hyaluronidase is reduced in the presence of low concentrations of a large number of "natural" and synthetic inhibitors.[2-11] Chondroitinsulfuric acid, a natural substrate of the enzyme, possesses in solution a configuration and charge[12,13] similar to that of anionic detergent micelles.[14] The experiments to be described below were undertaken in order to investigate the hypothesis that the inhibition of hyaluronidase by low molecular weight organic compounds is related to the micelle-forming properties of these compounds.

Experimental

Materials.—The highly purified sodium salts of straight chain sulfated fatty alcohols with, respectively, 8, 10, 12, 14 and 16 carbon atoms were obtained from M. L. Corrin of the Department of Chemistry, The University of Chicago. Sodium di-(2-ethylhexyl)-sulfosuccinate (aerosol OT) was purchased as a pure solid (99% purity claimed by American Cyanamid Corp.). A heparin preparation (lot no. 199) was obtained from Hynson, Westcott and Dunning, Inc. An ammonium salt of aurin tricarboxylic acid (ATA) was a specially purified (Eastman) preparation obtained from J. Schubert of Argonne National Laboratories. The *d*-α-tocopheryl phosphate (93% purity) was a gift by S. R. Ames of Distillation Products, Inc. Crystalline hexylresorcinol (m.p. 69°) was generously supplied by Winthrop-Stearns Co. The partially purified hyaluronidase[15] (bull testes) preparations as well as a hyaluronic acid[16] (human umbilical cord) preparation have been described.

Methods.—The turbidimetric assay[16] for hyaluronidase activity was used with substitution of the buffer employed for dilution of the enzyme. The method consisted essentially of the following steps: (1) incubation of 1 cc. of a known dilution of enzyme in 0.01 *M* phosphate buffer *p*H 7.0 and 0.45% sodium chloride with 1 cc. of hyaluronic acid in 0.3 *M* phosphate buffer *p*H 5.5 at 38° for 45 minutes; (2) addition of 10 cc. of acidified bovine serum albumin (Armour fraction V) solution to produce a turbidity; (3) measurement of the optical density, which is quantitatively related to the amount of hyaluronic acid remaining unde-

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