ISOLATION OF 6-HYDROXYOESTRADIOL-17β AND OESTRIOL AFTER INCUBATION OF OESTRADIOL-17β WITH RAT-LIVER SLICES

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SUMMARY

Following the incubation of oestradiol-17β with liver slices of male rats two metabolites more "polar" than oestradiol-17β were isolated and conclusively identified as 6-hydroxyo estradiol-17β and oestriol.

INTRODUCTION

In 1949, PEARLMAN AND DEMEIO[1] incubated oestradiol-17β hemisuccinate with rat-liver slices and obtained a comparatively large, strongly acidic phenolic fraction. The authors considered the possibility that this fraction might have contained oestriol. Recently, HAGOPIAN AND LEVY[2] reported the isolation of radioactive oestriol after incubation of 16-^{14}C-oestradiol-17β with a coarse brei of fresh liver obtained from male adult rats. Shortly afterwards, BREUER, NOCKE AND KNUPPEN[3] found that oestradiol-17β when incubated with rat liver slices was metabolized not only to oestriol but also to 6-hydroxyoestradiol-17β and 6-hydroxyoestrone. However, owing to the lack of material none of metabolites could be isolated. It is the purpose of the present paper to describe a large-scale experiment which led to the isolation of 6-hydroxyoestradiol-17β and oestriol after incubation of oestradiol-17β with rat-liver slices.

EXPERIMENTAL

Male Sprague-Dawley rats, average weight 160–200 g, were killed by decapitation followed by exsanguination. The livers were removed as rapidly as possible and covered with isotonic ice-cold saline until sliced. Liver slices were obtained by the method of DEUTSCH[4].

The procedures used for the paper chromatography were as described by BREUER AND NOCKE[5].

Spectrophotometric measurements were made in a 1.0 cm cell with a Beckman spectrophotometer model DU.

The melting points were determined with a microscope hot-stage. The same thermometer was used for all determinations, and the values given are uncorrected for emergent stem (*cf.*[6]).

Reference steroids. 6-Hydroxyoestradiol-17β (m.p. 247–249°) was prepared by the reduction of 6-oxooestradiol-17β with sodium borohydride. In earlier papers[7–9] the hydroxyl group at position 6 was designated 6 "β" but recently WINTERSTEINER AND MOORE[10] published experiments on the basis of which 6-hydroxyoestradiol-17β is tentatively assigned the 6"α" (quasi-equatorial) configuration. 6-Hydroxyoestradiol-17β 3-methyl ether was prepared from 6-hydroxyoestradiol-17β by treatment with dimethyl sulfate in alkaline solution at 37°. The product was recrystallized from benzene–light petroleum and melted at 109–110°. 6-oxooestrone (246–248°) was obtained after oxidation of oestrone acetate with CrO_3 in acetic acid followed by hydrolysis of 6-oxooestrone acetate with 2 *N* methanolic KOH. The 3-methyl ethers of 6-oxooestrone (m.p. 146–147°) and of oestriol (m.p. 158–160°) were prepared from the respective free compounds by treatment with dimethyl sulfate. Acetylation of oestriol yielded oestriol triacetate (m.p. 128°).

RESULTS

Incubation

Incubations were carried out in 150 ml conical flasks containing 20 ml of Krebs phosphate saline[11] containing 20 m*M* glucose, pH 7.4, 6 g of liver slices and 0.4 ml ethanolic solution of oestradiol-17β (25 mg/ml). Thus, in the final reaction mixture the tissue–steroid ratio was 600 : 1. The flasks were not stoppered but were shaken vigorously during the incubation for 120 min at 37°. In this manner, a total of 600 mg of oestradiol-17β was incubated with approx. 360 g of liver slices.

Lipid extracts

After incubation the saline and the tissue were extracted twice with equal volumes of an ether–chloroform mixture (3 : 1, v/v) and once with an equal volume of ether. The combined extracts were washed twice with one-quarter volume of water, dried over sodium sulphate and evaporated to dryness. The yellow residue was dissolved in 100 ml of 70% aqueous methanol; cholesterol and gummy impurities were removed by two washings with equal volumes of *n*-heptane. The aqueous methanol was then evaporated to dryness under reduced pressure. In order to separate the more "polar" material from the less "polar" one the semicrystalline residue was extracted 10 times with 100 ml portions of water. The aqueous extracts containing the more "polar" fraction were reextracted with equal volumes of benzene-light petroleum (1 : 1), and the combined aqueous extracts were then evaporated to 250 ml under reduced pressure. The aqueous phase was acidified with 15 ml of *N* HCl and thoroughly extracted with ether. The combined ether extracts (800 ml) were washed with 50 ml of 0.2 *M* $NaHCO_3$ and two 30 ml portions of water, dried over sodium sulphate and evaporated to dryness to yield the "polar" fraction (11 mg).

Methylation and chromatography

Previous experiments indicated that more than one substance was present in the "polar" fraction. An attempt to separate the single components from each other by chromatography on alumina failed, and it was therefore decided to attempt separation via the methyl ethers. The "polar" fraction was methylated with dimethyl sulphate as described by BROWN[12]. The methylated material was dissolved in benzene and then applied to an alumina column.

Investigation of fractions

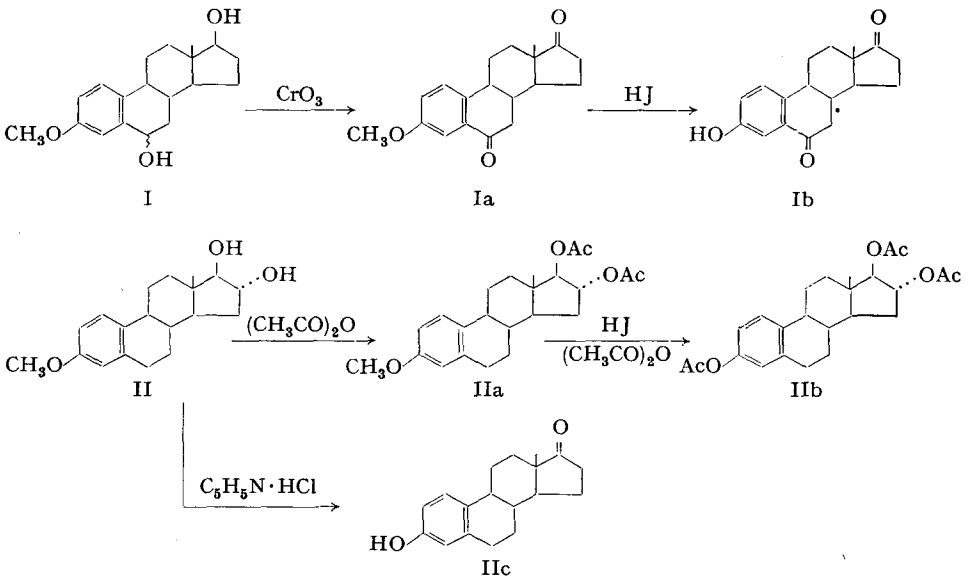
Fraction A. The fraction eluted with 20 ml of 1.4 % ethanol in benzene yielded 1.6 mg of colourless crystals (I), which were recrystallized from benzene–light petroleum and had m.p. 109°. After admixture with authentic 6-hydroxyoestradiol-17β 3-methyl ether the m.p. was not depressed. In 95 % ethanol solution I showed an u.v. absorption maximum at 280 mμ. For further identification, 1.3 mg of I were treated at room temperature for 3 h with a solution of CrO_3, 2.4 N with respect to oxygen, in 5 ml acetic acid. The excess of CrO_3 was destroyed with methanol, the solution diluted with water and then extracted with ether. 1.0 mg of colourless crystals (Ia) were obtained and recrystallized from light petroleum. Ia had a m.p. of 146° which was not depressed upon admixture with an authentic sample of 6-oxo-oestrone 3-methyl ether. The u.v. spectrum of Ia showed two maxima at 255 and 320 mμ and was identical with that of authentic 6-oxooestrone 3-methyl ether. Finally, Ia was demethylated using the method of HUFFMAN[13]. 1 ml of freshly distilled hydriodic acid was added to 0.5 mg of Ia dissolved in 1 ml of acetic acid. The mixture was heated for 5 min over a free flame, diluted after cooling with 20 ml cold aqueous sodium bisulphite and then extracted with ether. The material (Ib) obtained was subjected to paper chromatography in the system formamide-monochlorobenzene. After spraying the paper with FOLIN–CIOCALTEU reagent[14], a spot was detected which had the same R_F value as authentic 6-oxooestrone (0.23). The u.v. spectrum of Ib was identical with that of authentic 6-oxooestrone.

Fraction B. The fractions eluted with 30 ml of 3.0 % ethanol in benzene yielded 6.6 mg of colourless crystals (II) which gave a positive DAVID test[15]. After recrystallization from benzene the material melted at 158–160° and the mixed m.p. with oestriol 3-methyl ether was 158–159°. 3.3 mg of II were acetylated with acetic anhydride and pyridine; the diacetate (IIa) was demethylated by treatment with freshly distilled hydriodic acid as described by HUFFMAN[13]. After hydrolysis with methanolic KOH the material was again acetylated (IIb) and crystallized from methanol. After admixture with authentic oestriol triacetate (m.p. 128°) the m.p. was 127°. Another sample of II (3 mg) was converted to oestrone by heating it with 100 mg pyridine hydrochloride at 220° for 1.5 h[16]. The product obtained (IIc) was crystallized from ethanol and melted at 254–255°. After admixture with authentic oestrone the m.p. was not depressed. On paper chromatography in the system formamide–monochlorobenzene IIc showed the same R_F value as oestrone.

The reaction sequences establishing the identity of I with 6-hydroxyoestradiol-17β 3-methyl ether and of II with oestriol 3-methyl ether are shown in the reaction scheme.

DISCUSSION

The results reported here confirm and to some extent supplement the findings of HAGOPIAN AND LEVY[2] that oestradiol-17β can be transformed to oestriol in species other than man. The additional isolation of 6-hydroxyoestradiol-17β demonstrates that the hydroxylation of oestradiol-17β by rat liver slices is not confined to position 16 but also occurs at position 6 of the steroid molecule. It should be noted that 6-hydroxylation in the oestrogen series had previously been found with mouse liver preparations by MUELLER AND RUMNEY[7], and recently, 6-hydroxyoestrone has also been isolated from human urine by MARRIAN[17].



Reaction scheme.

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