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The Isolation of 16β-Hydroxyoestrone and 16-Oxo-oestradiol-17β from the Urine of Pregnant Women

By D. S. LAYNE and G. F. MARRIAN
Department of Biochemistry, University of Edinburgh

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While attempting to follow up the suggestion of Marrian & Bauld (1955) that 16-oxo-oestradiol-17β (3:17β-dihydroxyoestra-1:3:5-trien-16-one) might be the common metabolic precursor of oestriol (oestra-1:3:5-triene-3:16α:17β-triol) and 16-*epi*oestriol (oestra-1:3:5-triene-3:16β:17β-triol) Watson & Marrian (1955) reported the detection of a ketonic Kober chromogen in extracts of the urine of pregnant women which they provisionally identified as the sought-for 16-oxo-oestradiol-17β solely on partition-chromatographic and countercurrent-distribution evidence. Shortly afterwards Levitz, Spitzer & Twombly (1956) reported the detection by reverse isotope dilution of radioactive 16-oxo-oestradiol-17β in the urine of subjects after the administration of [16-^{14}C]oestradiol-17β ([16-^{14}C]-oestra-1:3:5-triene-3:17β-diol).

The following year the ketonic Kober chromogen detected in pregnancy urine by Watson & Marrian (1955) was isolated from the same source as a crystalline solid by Marrian, Watson & Panattoni (1957), and evidence was presented that the isolated product probably consisted of 16α-hydroxyoestrone (3:16α-dihydroxyoestra-1:3:5-triene-17-one) and a small proportion of 16-oxo-oestradiol-17β. It was furthermore suggested that the latter might have been an artifact produced from the former by rearrangement during the extraction of the phenolic fraction of the urinary extract by aqueous alkali from ether. The major component of the isolated product was subsequently identified with certainty as 16α-hydroxyoestrone by Marrian, Loke, Watson & Panattoni (1957), who also obtained evidence which apparently confirmed the earlier suggestion that the minor component in the product had been artifactually formed by rearrangement of 16α-hydroxyoestrone in alkaline solution. This evidence led the authors to conclude that pregnancy-urine extracts contain no 16-oxo-oestradiol-17β other than that which is formed artifactually in this way, and, because of this conclusion and of the difficulty in separating mixtures of 16α-hydroxyoestrone and 16-oxo-oestradiol-17β by crystallization they queried the validity of the claim of Levitz *et al.* (1956) to have detected 16-oxo-oestradiol-17β in urine extracts, although the method of extraction used by the latter authors seemed to exclude the possibility of artifactual formation of 16-oxo-oestradiol-17β.

Marrian, Watson & Panattoni (1957) and Marrian, Loke, Watson & Panattoni (1957) suggested that 16α-hydroxyoestrone formed from

oestrone (3-hydroxyoestra-1:3:5-trien-17-one) by 16α-hydroxylation might be the metabolic precursor of oestriol, and that similarly 16β-hydroxyoestrone (3:16β-dihydroxyoestra-1:3:5-trien-17-one) might be the precursor of 16-*epi*oestriol. At that time these suggestions were entirely speculative, but they appeared to be sufficiently plausible to justify an intensive search in pregnancy urine for 16β-hydroxyoestrone.

This search has now been successfully concluded, and it has also resulted in the isolation from pregnancy urine of substantial amounts of 16-oxo-oestradiol-17β by methods which as far as can be determined exclude the possibility that the latter was formed artifactually from either 16α-hydroxyoestrone or 16β-hydroxyoestrone. Accordingly, the earlier criticisms of the work of Levitz *et al.* (1956) are now unreservedly withdrawn.

RESULTS

Detection of 16α-hydroxyoestrone, 16β-hydroxyoestrone and 16-oxo-oestradiol-17β in pregnancy-urine extracts

Early attempts to separate 16β-hydroxyoestrone [prepared synthetically by the method of Biggerstaff & Gallagher (1957)], 16α-hydroxyoestrone and 16-oxo-oestradiol-17β by paper chromatography with systems of the Bush (1952) type by using aqueous methanol as stationary phase were entirely unsuccessful. However, acting on a suggestion made to us by Dr T. F. Gallagher of the Sloan-Kettering Institute, New York, we tried the system formamide/chloroform and obtained excellent separation of the three α-ketols from one another.

When ketonic–phenolic fractions, prepared from enzymically-hydrolysed pregnancy urine by the method of Marrian, Watson & Panattoni (1957), were chromatographed on paper in the formamide/chloroform system spots corresponding in mobilities to 16α-hydroxyoestrone and 16-oxo-oestradiol-17β were found, but nothing corresponding to 16β-hydroxyoestrone was detected. The possibility was therefore considered that any 16β-hydroxyoestrone which might originally have been present in the urine had undergone rearrangement to 16-oxo-oestradiol-17β during the working-up process. In particular the possibility was considered that rearrangement might have occurred during the 48 hr. incubation at pH 4·8 and 37° involved in the enzymic hydrolysis of the urine, during the Girard separation of the ketonic fraction or during the 10 min. exposure to alkali involved in the separation of the phenolic fractions. In experiments with synthetic 16β-hydroxyoestrone no rearrangement to 16-oxo-oestradiol-17β, as judged by paper chromatography, was observed after incubation in aqueous solution at pH 4·8 and 37° for 48 hr. or after standing overnight at room temperature in ethanolic acetic acid. However, complete rearrangement to 16-oxo-oestradiol-17β was found to occur after the 16β-hydroxyoestrone had stood for 10 min. at room temperature in N-sodium hydroxide solution.

In view of these findings a ketonic neutral-phenolic fraction of urine was prepared without the use of alkali and chromatographed on a Celite column. A portion of the fraction from the column expected to contain the α-ketolic oestrogen derivatives was chromatographed on paper and spots corresponding in their mobilities to 16α-hydroxyoestrone, 16β-hydroxyoestrone and, unexpectedly, 16-oxo-oestradiol-17β were detected. Areas of paper corresponding to these spots were cut out from a second chromatogram run simultaneously, and were separately eluted. Each of the three fractions thus obtained was treated with N-sodium hydroxide solution for 10 min. and, after recovery from the alkaline solutions in the usual way, these were rechromatographed on paper. The alkali treatment resulted in no change in mobility of the 16-oxo-oestradiol-17β-like material, a partial conversion of the 16α-hydroxyoestrone-like material and a complete conversion of the 16β-hydroxyoestrone-like material into products which had the same mobilities as 16-oxo-oestradiol-17β. These findings strongly suggested that the spots detected by paper chromatography in the extract were indeed due to 16α-hydroxyoestrone, 16β-hydroxyoestrone and 16-oxo-oestradiol-17β. However, because of the non-specificity of the Folin & Ciocalteu (1927) reagent used for detecting the spots on the paper chromatograms it was deemed necessary to attempt the isolation of the three α-ketols so that they could be characterized by orthodox methods.

Isolation of 16α-hydroxyoestrone, 16β-hydroxyoestrone and 16-oxo-oestradiol-17β from pregnancy-urine extracts

The procedure used for the isolation of the α-ketols was a scaled-up modification of that used for the detection of the compounds. The ketonic neutral-phenolic fraction from 200 l. of pregnancy urine was chromatographed on Celite columns. The fractions containing the α-ketols were chromatographed on a large number of sheets of paper. The three fractions obtained by eluting the appropriate areas of the papers, and which were believed to contain respectively the three α-ketols, were each purified by further paper chromatography and the final products acetylated.

The three acetates after purification were identified with certainty as 16α-hydroxyoestrone di-

acetate, 16β-hydroxyoestrone diacetate and 16-oxo-oestradiol-17β diacetate by their melting points, by mixed melting points with authentic specimens of the acetates and by their infrared spectra. The yields of the chromatographically purified products before acetylation were respectively 20·6, 6·2 and 13·1 mg.

EXPERIMENTAL

Methods and Materials

Paper-chromatographic methods. Strips of Whatman no. 42 paper 34 cm. × 12·5 cm. were extracted in a Soxhlet apparatus for 3 days with a mixture of methanol and chloroform. After drying they were immersed in a 2:1 (v/v) methanol–formamide mixture, blotted with filter paper between two sheets of plate glass and dried in a horizontal position at 37° for 45 min.

Analytical chromatograms were carried out by applying to the paper methanolic solutions of the extracts or of the pure compounds in amounts containing about 10–25 μg. of Kober chromogen. After equilibration in the tanks for at least 1 hr. at 18±2° the chromatograms were developed with chloroform for 10–12 hr. The spots were located by spraying the papers with diluted Folin–Ciocalteu phenol reagent and then hanging the papers in a tank over ammonia solution. After a 12 hr. run the distances from the starting line of the 16α-hydroxyoestrone, 16-oxo-oestradiol-17β and 16β-hydroxyoestrone spots were respectively about 26, 23 and 19 cm.

Preparative-scale chromatograms were carried out by applying across the whole width of each paper about 15 mg. of a urinary fraction in methanolic solution in a streak about 1 cm. wide. After development of the chromatogram, strips 1 cm. in width were cut from each side of each paper and the positions of the oestrogen derivatives located on these strips by the Folin–Ciocalteu reagent. The appropriate areas were then cut out from the main part of each paper, and these thoroughly extracted with methanol at room temperature. After evaporation of the methanolic extracts formamide was removed from the eluted fractions by distribution between ether and water; the ether extracts were dried over sodium sulphate and evaporated to dryness.

Melting points. The melting points of the acetates of the α-ketols were determined with a microscope hot-stage, while those of the α-ketols themselves were determined in sealed evacuated capillaries. All melting points were determined with the same thermometer and the values given are uncorrected for emergent stem.

Reference compounds. 16α-Hydroxyoestrone, 16α-hydroxyoestrone diacetate, 16-oxo-oestradiol-17β and 16-oxo-oestradiol-17β diacetate were prepared as described previously (Marrian, Watson & Panattoni, 1957; Marrian, Loke, Watson & Panattoni, 1957). 16β-Hydroxyoestrone diacetate, m.p. 139–141°, $[\alpha]_D^{15}$ +132° in ethanol (*c*, 0·494), was prepared by our colleague Mr K. H. Loke by a method based on that of Biggerstaff & Gallagher (1957). Hydrolysis of this by the method used for the hydrolysis of 16α-hydroxyoestrone diacetate yielded 16β-hydroxyoestrone, m.p. 237–239° (change of form 232°). As judged by column and paper chromatography this preparation was contaminated with about 5% of 16-oxo-oestradiol-17β.

Isolation of 16α-hydroxyoestrone, 16β-hydroxyoestrone and 16-oxo-oestradiol-17β from the urine of pregnant women

Late-pregnancy urine was hydrolysed enzymically in batches of about 16 l. as previously described (Marrian, Watson & Panattoni, 1957). Before its extraction once with an equal volume of ether, Bradosol (a 5% solution of β-phenoxyethyldimethyldodecylammonium bromide; Ciba Laboratories Ltd.) (1 ml./l.) was added to facilitate the breaking of emulsions. The ether extract was washed four times with 5% (w/v) $NaHCO_3$ solution and twice with water, dried over Na_2SO_4 and evaporated to dryness. The neutral-phenolic material from each batch was treated overnight at room temperature with 4 g. of trimethylammoniumhydrazide chloride in 40 ml. of ethanol and 8 ml. of acetic acid, and the ketonic fraction separated in the usual way.

Combined ketonic fractions corresponding to about 50 l. of urine were each chromatographed in a 2 cm. diameter column on 120 g. of Celite with the system 70% (v/v) methanol in water–20% (v/v) hexane in benzene. The first 240 ml. of eluate was rejected and the next 800 ml. containing the α-ketolic oestrogen derivatives was evaporated to dryness. The total weight of chromatographically concentrated material obtained from 200 l. of urine was 1·23 g.

This material was chromatographed on 80 sheets of paper in the formamide/chloroform system. After this purification the crude 16α-hydroxyoestrone, 16β-hydroxyoestrone and 16-oxo-oestradiol-17β fractions weighed respectively 121·2, 21·7 and 44·0 mg.

The first of these fractions was rechromatographed on eight sheets of paper, after which 20·6 mg. of material was obtained. The second fraction after rechromatography on two sheets of paper yielded 6·2 mg., while the third after rechromatography on three sheets yielded 13·1 mg. All three fractions were semicrystalline after rechromatography, but the second (16β-hydroxyoestrone) was heavily contaminated by a yellow oil.

The 16α-hydroxyoestrone fraction was acetylated overnight at room temperature with acetic anhydride and pyridine. After one crystallization from ethyl acetate–hexane the acetate melted at 169–172° and had $[\alpha]_D^{16}$ +141° in ethanol (*c*, 0·508). After admixture with authentic 16α-hydroxyoestrone diacetate (m.p. 171–172°, $[\alpha]_D^{19}$ +149°) the m.p. was 171–172·5°. The infrared spectrum of the acetate in carbon disulphide solution was identical with that of the authentic sample.

The 16β-hydroxyoestrone fraction after acetylation was still heavily contaminated with a yellow oil, and attempts to crystallize it from ethyl acetate–hexane and methanol were unsuccessful. It was, therefore, chromatographed on a column of 5 g. of aluminium oxide [acid-washed; activated at 120°; deactivated with 5% (v/w) water] with 50% (v/v) hexane in benzene and benzene as eluents. The benzene eluate yielded 4·1 mg. of a gum which yielded 2·1 mg. of white crystals (m.p. 138–141°) on crystallization from methanol. After admixture with authentic 16β-hydroxyoestrone diacetate (m.p. 139–141°) the m.p. was 139–141°. The infrared spectra of the two acetates were identical.

The 16-oxo-oestradiol-17β fraction was acetylated, and after crystallization from ethyl acetate–hexane yielded

7·1 mg. of white crystals which had m.p. 133·5–135·5° and $[\alpha]_D^{18}$ −71° in ethanol (*c*, 0·536). The mixed m.p. with authentic 16-oxo-oestradiol-17β diacetate [(m.p. 132·5–134°; $[\alpha]_D^{17}$ −64° in ethanol (*c*, 0·546)] was 130·5–133·5°. The infrared spectra of the two acetates were identical.

DISCUSSION

The isolation of 16β-hydroxyoestrone from the urine of pregnant women described here is the first reported isolation of this oestrogen derivative from a natural source. In view of the ease with which this compound undergoes rearrangement to 16-oxo-oestradiol-17β it seems unlikely that the isolated material was an artifact.

This isolation provides further support for the speculative scheme for the biogenesis of oestriol and 16-*epi*oestriol advanced by Marrian, Watson & Panattoni (1957) and Marrian, Loke, Watson & Panattoni (1957). The two hypothetical intermediates between oestrone and oestriol and 16-*epi*oestriol have now both been shown to be present in pregnancy urine, and it has previously been shown (Brown & Marrian, 1957) that 16α-hydroxyoestrone undergoes metabolic reduction to oestriol in man. It now remains to be shown whether oestrone can undergo 16-hydroxylation to form 16α-hydroxyoestrone and 16β-hydroxyoestrone, and whether the latter can give rise to 16-*epi*oestriol by metabolic reduction.

The isolation of 16-oxo-oestradiol-17β from pregnancy urine by a process which, as far as could be determined, could not have led to the artifactual formation of the compound from either 16α-hydroxyoestrone or 16β-hydroxyoestrone shows that Marrian, Loke, Watson & Panattoni (1957) were incorrect in their view that pregnancy-urine extracts contain no 16-oxo-oestradiol-17β other than that which is formed as an artifact during the isolation process.

As far as can be judged from the yields of the compounds isolated in the course of the present work, 16-oxo-oestradiol-17β must be an oestrogen metabolite quantitatively comparable with 16α-hydroxyoestrone. It is therefore of some importance to consider the metabolic relationships of 16-oxo-oestradiol-17β to the other ring-D-disubstituted oestrogen metabolites. Levitz, Spitzer & Twombly (1958) have recently shown that [16-^{14}C]-oestriol administered to human subjects gives rise to radioactive 16-oxo-oestradiol-17β and 16-*epi*oestriol in the urine. These important findings show clearly that oestriol undergoes partial oxidation at C-16 in the body to form 16-oxo-oestradiol-17β, and that the latter can undergo metabolic reduction, with the formation of 16-*epi*oestriol. The present authors nevertheless believe that the 16-*epi*oestriol may also be formed from oestrone via 16β-hydroxyoestrone, although proof that this is so has yet to be obtained.

Note added in proof. Since the submission of this paper Brown, Fishman & Gallagher (1958) have reported the detection of radioactive 16β-hydroxyoestrone by reverse isotope dilution in the urine of subjects treated with [16-^{14}C]oestradiol-17β.

SUMMARY

1. A ketonic neutral-phenolic fraction obtained from enzymically hydrolysed late-pregnancy urine was subjected to partition chromatography and then paper chromatography. By means of the Folin–Ciocalteu reagent spots corresponding in their mobilities to those of 16α-hydroxyoestrone, 16-oxo-oestradiol-17β and 16β-hydroxyoestrone were detected.

2. By using similar methods on a large scale three semicrystalline concentrates believed to contain the three α-ketolic oestrogen derivatives respectively were prepared from 200 l. of late-pregnancy urine.

3. Acetylation of these concentrates yielded three crystalline acetates which were identified as those of 16α-hydroxyoestrone, 16-oxo-oestradiol-17β and 16β-hydroxyoestrone by melting points, mixed melting points and infrared spectrometry.

4. This demonstration of the occurrence of 16β-hydroxyoestrone in urine is compatible with the previously expressed view (Marrian, Watson & Panattoni, 1957; Marrian, Loke, Watson & Panattoni, 1957) that this compound is the intermediate in the metabolic conversion of oestrone into 16-*epi*oestriol.

5. Since the isolation procedure employed was unlikely to have caused artifactual formation of 16-oxo-oestradiol-17β by rearrangement of 16α-hydroxyoestrone or 16β-hydroxyoestrone, this work supports the view of Levitz, Spitzer & Twombly (1956) that 16-oxo-oestradiol-17β is a naturally occurring oestrogen metabolite.

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Phosphate Esters in the Uterus

By J. M. CAWKWELL
Department of Biochemistry, University of Cambridge

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Hollmann (1949) observed that more than half the acid-soluble phosphate esters extracted from cow uterus resisted acid hydrolysis. After 10 hr. in N-HCl at 100°, 72·5% of the organic phosphate was still unhydrolysed, and he suggested that the acid-resistant ester was phosphoglyceric acid.

In a preliminary experiment on an extract from rat uteri, 35% of the acid-soluble phosphate resisted N-HCl at 100° for 3 hr., but very little phosphoglyceric acid was detectable by the enzymic method of Slater (1953). Since Awapara, Landua & Fuerst (1950) have demonstrated the presence of phosphorylethanolamine in extracts of rat uterus, it was decided to make a further study of the phosphate esters in the uterus. Extracts were prepared from non-pregnant uteri of a number of mammalian species, and from pregnant and post-partum rabbit uteri. Phosphate analyses were carried out on all the extracts. The extracts from pregnant and post-partum rabbit uteri were also investigated by paper chromatography.

EXPERIMENTAL

Extracts were prepared in three ways.

(1) A method described by Menkes & Csapo (1952) was used. Rats in oestrus were anaesthetized with Nembutal and the uteri dissected out. Four uteri were extracted together. After 20 min. in ice-cold oxygenated saline, they were blotted dry, frozen with liquid nitrogen and powdered in a mortar. The powder was extracted with 10 vol. of 5% (w/v) trichloroacetic acid, and, after being left for 30 min. at 0°, the extract was filtered.

(2) The uterus was removed from a non-pregnant anaesthetized rabbit and, after the internal surface had been gently scraped, the tissue was frozen in liquid nitrogen. It was ground to a powder and extracted with 5% trichloroacetic acid as in method (1). This method was also used for non-pregnant pig and cow uteri which were brought from the slaughter house packed in ice, immediately after the death of the animal. Human uterus, which was obtained immediately after removal at operation, was extracted in this way.

(3) Extracts were prepared from the uteri of rabbits in the fourth week of pregnancy and just post partum, by the method of Dawson (1955*a*). In this method the danger of the liberation of glycerylphosphorylcholine and glyceryl-phosphorylethanolamine from the tissue phospholipids is diminished by the substitution of boiling water for trichloroacetic acid.

The animals were stunned and the uteri removed and packed in ice. Further steps were carried out in the cold. Remnants of the placenta were gently removed with a blunt scalpel, care being taken to remove as little as possible of the endometrium. After freezing in liquid nitrogen, the uteri were powdered. The powder was transferred to a beaker, extracted with 4 vol. of boiling water and kept in a boiling-water bath for 2 min. Absolute ethanol (6 vol.) was added and the solution was centrifuged. The supernatant was shaken with chloroform to extract the ethanol, and the aqueous layer was recentrifuged and passed through a column of Amberlite IRC 50 H^+ to remove metal ions. It was then shaken with 2 vol. of butan-2-ol to remove any turbidity and centrifuged. The supernatant was neutralized and dried *in vacuo*, the temperature being kept below 50°. The residue was dissolved in a known volume of water. Each rabbit uterus was extracted separately, and two or three extracts were combined before the material was dried *in vacuo*.

One uterus from a pregnant rabbit was extracted in two different ways: with water and with trichloroacetic acid. The frozen powder was divided into two parts, one part extracted with trichloroacetic acid as in method (1) and the other part with boiling water as in method (3).

Phosphoglyceric acid estimation. The enzymic method of Slater (1953) was used to estimate the phosphoglyceric acid in the trichloroacetic acid extracts from non-pregnant pig, rat, cow and human uteri, and in the water extract from pregnant rabbit uterus. Since this method does not distinguish phosphoglyceric acid from adenosine triphosphate and adenosine diphosphate, the trichloroacetic acid extracts were left overnight at room temperature to allow the hydrolysis of the acid-labile phosphates to occur.