The Isolation of 6 α -Hydroxyoestrone from the Urine of Pregnant Women

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1. In view of previous experiments *in vitro* and *in vivo*, in which 6-hydroxylation of phenolic steroids by various tissues was demonstrated, an attempt was made to isolate 6-hydroxy oestrogens from the urine of pregnant women. 2. During this work it was found that free 6 α - as well as 6 β -hydroxylated oestrogens were extremely unstable under acidic conditions (pH < 3); it was therefore necessary to establish experimental conditions under which no rearrangement of the hydroxyl group would take place. 3. By avoiding acid steps during the experimental procedures, a ketonic-phenolic fraction was obtained from enzymically hydrolysed late-pregnancy urine that was subjected to paper chromatography in various systems. 4. By using similar methods on a large scale and partition chromatography on Celite columns, a crystalline material was obtained from 100l. of late-pregnancy urine that was identified as 6 α -hydroxyoestrone by various chemical reactions and by infrared spectroscopy.

During recent years, the biogenesis and metabolism of 6-substituted phenolic steroids have been extensively studied (see Breuer, 1962). It was found that 6 α -hydroxylation of oestrone and oestradiol-17 β occurs with liver tissue preparations of mouse (Mueller & Rumney, 1957), rat (Breuer, Nocke & Knuppen, 1959; Breuer & Knuppen, 1960; Breuer, Knuppen & Pangels, 1962) and human foetus (Breuer, Knuppen, Ortlepp, Pangels & Puck, 1960a), with human ovary (Breuer, Knuppen & Pangels, 1960b), with ox adrenal (Knuppen, Behm & Breuer, 1964) and during perfusion of human placenta (Cedard & Knuppen, 1965). From experiments with 6-oxo-oestradiol-17 β , it was demonstrated that, like rat liver (Breuer *et al.* 1962), human liver also contains a 6 α - and a 6 β -hydroxy steroid oxidoreductase (Breuer, Knuppen & Pangels, 1961). A similar observation was made after injection of 6-oxo-oestrone into man; 6 α - as well as 6 β -hydroxylated oestrone and oestradiol-17 β were formed as metabolites *in vivo* (Breuer & Breuer, 1965).

Thus far, no 6-oxo or 6-hydroxy phenolic steroids have yet been shown with certainty to occur in urine, but it has been suggested that the Kober chromogen in the urine of pregnant women, referred to as KC-6B (Loke, Marrian & Watson, 1959), may be a 6-hydroxyoestrone (Marrian & Sneddon, 1960). In view of the results of the studies *in vitro* described above, the evidence suggesting the presence of a 6-hydroxylated oestrogen in

human urine appeared to be so convincing that it was decided to attempt its isolation and identification. During this work, it soon became clear that 6 α - and 6 β -hydroxylated oestrogens readily undergo epimerization and dehydration under acidic conditions. It therefore seemed necessary to establish experimental conditions that would permit definite proof of the configuration of the 6-hydroxylated oestrogen isolated from urine.

METHODS

Reference compounds. 6 α -Hydroxyoestrone was prepared by the method of Knuppen & Breuer (1961) and 6 β -hydroxyoestrone by the method of Breuer *et al.* (1962). 6 α - and 6 β -Hydroxyoestradiol-17 β were prepared as described by Wintersteiner & Moore (1959). 6-Oxo-oestrone was obtained by the method of Schwenk (1942); 6-dehydro-oestradiol-17 β was prepared by dehydration of 6 α -hydroxy-oestradiol-17 β , and 6-dehydro-oestrone by oxidation of 6-dehydro-oestradiol-17 β with chromic acid. 6 α - and 6 β -Methoxyoestrone were obtained by treatment of 6 α -hydroxyoestrone with methanolic *N*-HCl for 1 hr. at 37°. Under these conditions, the 6 α -hydroxylated oestrogens yielded approx. 40% of 6 α -methoxy and 60% of 6 β -methoxy oestrogens.

Paper-chromatographic methods. Strips of Schleicher und Schüll 2043b Mgl paper (45 cm. $\times$ 15 cm.) were immersed in methanol-formamide (1:1, v/v), blotted with filter paper and dried in a horizontal position at room temperature for 30 min. All chromatograms were equilibrated for at least 1 hr. at 22 $\pm$ 2°. The chromatograms were developed with chloroform, chlorobenzene, chlorobenzene-ethyl acetate

(3:1, v/v) or chloroform-ethyl acetate (5:1, v/v) (Knuppen, 1962). One solvent system of the Bush (1952) type was employed: benzene-light petroleum (b.p. 40–60°)-methanol-water (4:6:7:3, by vol.).

Preparative-scale chromatograms were carried out by applying about 5mg. of a urinary fraction in methanolic solution in a streak about 5.0 cm. wide. After development of the chromatograms, strips 1 cm. wide with pure reference compounds were cut from each side from each paper and the positions of the oestrogens located on these strips by the Folin-Ciocalteu reagent (Mitchell & Davies, 1954). The appropriate areas were then cut out from the main part of each paper and these thoroughly extracted with methanol at room temperature.

Column partition chromatography. Partition chromatography on Celite columns with the solvent system benzene-methanol-water (2:1:1, by vol.) was carried out in a constant-temperature room (25°). For purification, Celite 535 (Johns Manville International Corporation, New York, N.Y., U.S.A.) was covered with hot conc. HCl for 24 hr., filtered, washed thoroughly with water until free from chloride and dried at 120°. The Celite was then extracted in a Soxhlet apparatus for 48 hr. with benzene-ethyl acetate-methanol (1:1:1, by vol.).

Melting points. The melting points were determined with a microscope hot-stage and are corrected.

Miscellaneous. Ultraviolet spectra were recorded in methanol with a Beckman model DK2-A spectrophotometer; infrared spectra were measured by using a KBr disk with a Beckman model IR8 spectrophotometer. Nuclear-magnetic-resonance spectra were recorded on a Varian A 60 analytical spectrometer. Optical rotations were determined in dioxan. The Kober reaction was carried out by the methods of Brown (1952) and Bauld (1954), with the optimum conditions as described by Nocke (1961). Elementary analyses were carried out by Ilse Beetz, Kronach, Germany.

Hydrolysis and extraction of urine. Specimens (11.) of late-pregnancy urine were acidified to pH 4.8 with acetic acid, buffered to this pH by the addition of 0.1 vol. of α -acetate buffer and incubated at 37° with an extract from *Helix pomatia* [2 ml., containing 100 000 Fishman units (Talalay, Fishman & Huggins, 1946) of β -glucuronidase and 50 000 Whitehead units (Whitehead, Morrison & Young, 1952) of sulphatase/ml.] for a period of 48 hr.; half of the total amount of enzyme used was added initially and half after 24 hr. The hydrolysed mixture was extracted twice with 0.67 vol. of ether; the extracts were combined and washed once with 0.1 vol. of 8% (w/v) NaHCO_3 and then extracted twice with 0.17 vol. of N-NaOH . The NaOH extracts were partly neutralized with 2N- H_2SO_4 and buffered to pH 7–8 with 8% (w/v) NaHCO_3 . The neutralized solution was extracted twice with 1 vol. of ether, and the extract was washed twice with 0.25 vol. of water, dried over Na_2SO_4 and evaporated to dryness. Phenolic fractions obtained in this way were stored at -10° in ethanolic solution until 100l. of urine had been processed. They were then combined and evaporated to dryness under reduced pressure. The residue was acetylated at room temperature with acetic anhydride (35 ml.) in pyridine (30 ml.) for 24 hr. After dilution with water (300 ml.), the mixture was extracted twice with ether (500 ml.). The ether extracts were combined and washed in the usual way with 2N- H_2SO_4 , 8% (w/v) NaHCO_3 and

water. The ethereal phase was dried over Na_2SO_4 and evaporated to dryness. The residue was treated at room temperature for 24 hr. with trimethylammonium hydrazide chloride (Girard T reagent) (5g.) in ethanol (70 ml.) and acetic acid (12 ml.). The acetylated ketonic and non-ketonic fractions were separated in the usual way. The acetylated ketonic fraction was hydrolysed with methanolic 5% (w/v) KOH at room temperature under nitrogen for 24 hr. After dilution with water the mixture was neutralized to pH 7–8 with N- H_2SO_4 and then extracted twice with 0.67 vol. of ether; the combined extracts were washed with 0.2 vol. of 8% (w/v) NaHCO_3 and twice with 0.2 vol. of water, dried over Na_2SO_4 and evaporated to dryness (ketonic-phenolic fraction).

RESULTS

Studies on the stability of the 6-hydroxyl group. In preliminary experiments evidence was obtained indicating that the 6-hydroxyl group of 6α - as well as of 6β -hydroxyoestrone (and oestradiol-17 β) undergoes epimerization when the steroids are treated with acidic solutions. For the investigation of the configuration of the 6-hydroxyl group of the naturally occurring 6-hydroxyoestrone it was clearly necessary to establish experimental conditions under which no epimerization of the 6-hydroxyl group would take place during the isolation procedure. Therefore experiments were carried out in which the processes of epimerization, and also of dehydration, of 6-hydroxylated oestrogens were studied in detail. In this connexion, a relevant observation of Balant & Ehrenstein (1952) is that 6β -acetoxy- Δ^4 -3-oxo steroids are epimerized to the more stable 6α -acetoxy- Δ^4 -3-oxo compounds by dry hydrogen chloride in chloroform containing a small amount of ethanol; they explained the epimerization by the formation of an intermediary 6-acetoxy- $\Delta^{3,5}$ -dien-3-ol.

Treatment of 6α - and 6β -hydroxylated oestrogens with aqueous hydrochloric acid. 6α -Hydroxyoestrone (2 mg.) was dissolved in dimethylformamide (0.2 ml.). After dilution with water (9 ml.), aqueous concentrated hydrochloric acid (1 ml.) was added. The mixture was kept for 1 hr. at 37° and then extracted twice with 1 vol. of ethyl acetate. The combined extracts were washed with 8% (w/v) sodium hydrogen carbonate and water, dried over anhydrous sodium sulphate and evaporated to dryness. After chromatography on formamide-impregnated paper with chlorobenzene-ethyl acetate (3:1, v/v), three phenolic substances were detected whose identity with 6-dehydro-oestrone, 6α -hydroxyoestrone and 6β -hydroxyoestrone was proved by their ultraviolet and infrared spectra. Similar findings were obtained after treatment of 6β -hydroxyoestrone with aqueous hydrochloric acid. It should be noted that epimerization and dehydration of the 6-hydroxyl group were not

Table 1. *Oestrogens found after treatment of 6 α - and 6 β -hydroxyoestrone (100 μ g.) with aqueous N-hydrochloric acid (1 ml.) at 37° for various times*

The oestrogens were separated on formamide-impregnated paper in the system chlorobenzene-ethyl acetate (3:1, v/v); quantitative determinations were carried out by the Kober reaction (Nocke, 1961). Recoveries of the individual steroids were between 80 and 90%.

Oestrogen treated with HCl	Duration of treatment (hr.)	Percentage of oestrogen found		
		6 α -Hydroxy- oestrone	6 β -Hydroxy- oestrone	6-Dehydro- oestrone
6 α -Hydroxyoestrone	0	100	0	0
	1	35	60	5
	12	0	0	100
6 β -Hydroxyoestrone	0	0	100	0
	1	35	60	5
	12	0	0	100

confined to the 6-hydroxylated oestrones but also occurred with 6 α - and 6 β -hydroxyoestradiol-17 β . The quantitative results of experiments with 6 α - and 6 β -hydroxyoestrone are given in Table 1. When 6 α - and 6 β -hydroxylated oestrone were exposed to aqueous hydrochloric acid for 12 hr., only the corresponding 6-dehydro compound could be detected (see Table 1). Similar qualitative and quantitative results were obtained with 6 α - and 6 β -hydroxyoestradiol-17 β .

Treatment of 6 α - and 6 β -hydroxylated oestrogens with methanolic hydrochloric acid. 6 α -Hydroxy-oestrone (10 mg.) was dissolved in methanol (9 ml.) and aqueous concentrated hydrochloric acid (1 ml.) added. The mixture was kept for 1 hr. at 37°. After dilution with water (150 ml.), the mixture was twice extracted with 1 vol. of ether. The combined extracts were washed with 8% (w/v) sodium hydrogen carbonate and water, dried over anhydrous sodium sulphate and evaporated to dryness. A sample was chromatographed on formamide-impregnated paper with chlorobenzene, and three phenolic compounds were detected, one of which was formed in only small amounts and identified as 6-dehydro-oestrone. The steroid mixture was chromatographed in benzene on a column of 20 g. of neutral alumina, elution being carried out with 50 ml. portions of 0.25% ethanol in benzene (300 ml.), 0.4% ethanol in benzene (400 ml.) and 0.8% ethanol in benzene (500 ml.). The fractions eluted by 0.25% ethanol in benzene were combined and yielded 0.48 mg. of 6-dehydro-oestrone. On evaporation the fractions eluted by 0.4% ethanol in benzene yielded fraction A-1 (3.5 mg.), and the fractions eluted by 0.8% ethanol in benzene yielded fraction B-1 (5.8 mg.).

Fraction A-1 was crystallized from ethyl acetate and had m.p. 226–228°, not depressed after admixture with authentic 6 α -methoxyoestrone (m.p. 226–228°). Fraction A-1 gave C and H

analyses in excellent agreement with those required for a compound of the formula C₁₉H₂₄O₃. The ultraviolet, infrared and nuclear-magnetic-resonance spectra as well as the optical rotation [α]_D²¹ + 150° (c 0.501 in dioxan) were identical with those of authentic 6 α -methoxyoestrone.

Fraction B-1 was crystallized from ethyl acetate and had m.p. 184.5–186°, not depressed after admixture with authentic 6 β -methoxyoestrone (m.p. 185–186.5°). Analysis of fraction B-1 gave C and H values in good agreement with those for a compound of the formula C₁₉H₂₄O₃. The ultraviolet, infrared and nuclear-magnetic-resonance spectra as well as the optical rotation [α]_D²¹ + 133° (c 0.495 in dioxan) were identical with those of authentic 6 β -methoxyoestrone.

When 6 β -hydroxyoestrone (10 mg.) was treated with hydrochloric acid in methanol in the same way as 6 α -hydroxyoestrone, again three reaction products were obtained that were isolated and identified as 6-dehydro-oestrone (0.45 mg.), 6 α -methoxyoestrone (3.8 mg.) and 6 β -methoxyoestrone (5.7 mg.) in the same manner as described above.

Isolation and identification of 6 α -hydroxyoestrone. The ketonic-phenolic fraction, prepared from enzymically hydrolysed pregnancy urine, was subjected to preparative-scale paper chromatography on 30 sheets of formamide-impregnated paper with chloroform as mobile phase. The areas corresponding in mobility to 6 α -hydroxyoestrone (1.9 cm./hr.) were eluted with methanol. The 6 α -hydroxy-oestrone-like material was distributed between ether and water to remove residues of formamide. The ethereal phase was dried over anhydrous sodium sulphate and evaporated to dryness. The yellowish oil obtained was then chromatographed on 20 sheets of formamide-impregnated paper with chlorobenzene-ethyl acetate (3:1, v/v) for 10 hr. The 6 α -hydroxyoestrone-like material yielded three phenolic substances (I–III) whose mobilities were

similar to those of 6 α -hydroxyoestrone (I; 3.4 cm./hr.), 6 β -hydroxyoestrone (II; 2.9 cm./hr.) and 15 α -hydroxyoestrone (III; 4.5 cm./hr.). After elution with methanol, substance (I) was again distributed between ether and water, and the residue of the ether extract subjected to preparative-scale chromatography on 20 sheets of paper with benzene–light petroleum (b.p. 40–60°)–methanol–water (4:6:7:3, by vol.). The presence of two phenolic substances (Ia and Ib) was revealed; compound (Ia) showed the same mobility (21 cm./20 hr.) as 6 α -hydroxyoestrone, whereas compound (Ib) had a mobility of 18 cm./20 hr. The area containing substance (Ia) was eluted with methanol and chromatographed on a small column (0.8 cm. diam.) of Celite (6g.) with benzene–methanol–water (2:1:1, by vol.); 20 successive 2 ml. fractions of the eluate were collected and evaporated to dryness. Fractions 9–12 contained 3 mg. of white crystals.

The infrared spectrum of the crystalline material (Ia) was identical with that of authentic 6 α -hydroxyoestrone; the ultraviolet spectrum in methanol showed two maxima at 222 and 282 m μ and a shoulder at 287 m μ . For further identification, compound (Ia) was acetylated with acetic anhydride and pyridine at room temperature. The infrared spectrum of the acetylated material was identical with that of authentic 6 α -hydroxyoestrone diacetate. Another portion of substance (Ia) was reduced with sodium borohydride in methanol. The compound obtained had the same mobility (21 cm./15 hr.) as authentic 6 α -hydroxyoestradiol-17 β on formamide-impregnated paper with chloroform–ethyl acetate (5:1, v/v). Oxidation of compound (Ia) with chromic acid in acetone (Iriarte, Ringold & Djerassi, 1958) at 0° yielded a substance that was indistinguishable from 6-oxo-oestrone as judged by paper chromatography (a) with benzene–light petroleum (b.p. 40–60°)–methanol–water (4:6:7:3, by vol.) and (b) on formamide-impregnated paper with chlorobenzene. The ultraviolet spectrum of the oxidation product in methanol showed three maxima at 222, 255.5 and 325 m μ , and was identical with that of authentic 6-oxo-oestrone. When compound (Ia) was treated with N-hydrochloric acid in methanol under reflux for 1 hr., the product thus formed showed the same paper-chromatographic behaviour and the same ultraviolet spectrum (λ_{max} in methanol at 222, 263 and 304 m μ ; shoulder at 273 m μ) as authentic 6-dehydro-oestrone.

During the work-up procedure, the isolated material was only briefly exposed to acid, and it seemed unlikely that any 6 α - or 6 β -hydroxyoestrone would have undergone any transformation. Nevertheless, authentic samples of the two compounds were treated in the same way as the material

isolated from urine, and they were recovered unchanged.

It is considered that completely satisfactory proof for the identity of substance (Ia) and 6 α -hydroxyoestrone has been obtained.

Concentration of 6 α -hydroxyoestrone in pregnancy urine. Preliminary experiments were carried out on six different 24 hr. urine specimens collected during late pregnancy. It was found that the amounts of 6 α -hydroxyoestrone excreted varied considerably from one urine to another, the highest concentration observed in two urines being 150 $\mu\text{g.}/24\text{ hr.}$ (corrected for experimental losses). However, the quantitative significance of the 6-substituted oestrogens in pregnancy urine cannot as yet be assessed on the basis of the available information.

DISCUSSION

The results reported here show conclusively the presence of 6 α -hydroxyoestrone in the phenolic–ketonic fraction of enzymically hydrolysed late-pregnancy urine. This finding is not surprising in view of the results of previous experiments *in vitro* in which 6 α -hydroxylation of oestrone and oestradiol-17 β was shown to occur in human foetal liver (Breuer *et al.* 1960a) and in human placenta (Cedard & Knuppen, 1965). The question arises whether the formation and excretion of 6 α -hydroxyoestrone (and possibly 6 α -hydroxyoestradiol-17 β) has any significance under physiological conditions. The introduction of a hydroxyl group at C-6 in the oestrogen molecule greatly increases the relative solubility in water (Marrian & Sneddon, 1960), and it might well be that the physiological significance of the 6-substituted phenolic steroids is related to this characteristic.

In view of the finding that 6 α - and 6 β -hydroxyoestrone readily undergo rearrangement in acidic solution, it may be asked whether the 6 α -hydroxyoestrone isolated was formed as an artifact from 6 β -hydroxyoestrone, possibly present in pregnancy urine. However, this possibility can be ruled out, since the isolation process was carried out in such a way that 6 β -hydroxyoestrone, if present at all, could not be epimerized to the 6 α -hydroxyl compound.

The epimerization of the 6-hydroxylated phenolic steroids in acidic solutions described here can easily be explained by the presence of the phenolic ring A in α -position to C-6 of the steroid molecule. The influence of acidic solutions on 6 α - and 6 β -hydroxylated oestrogens will result first in the formation of a stable carbonium ion. The remaining hydrogen atom in position 6 is now in the same plane as the phenolic ring A and no longer fixed in the α - or β -position. This makes it possible for nucleophilic agents, such as water or methanol, to attack the

carbonium ion from the α - or β -side of the molecule. In the second step of the reaction, a monomolecular nucleophilic substitution (S_N1 mechanism) takes place: in aqueous acidic solution, both the 6 α - and 6 β -hydroxy oestrogens are formed, whereas in methanolic acidic solutions both the 6 α - and 6 β -methoxy oestrogens arise. These theoretical considerations are in agreement with the results described above. It should be noted that, as well as the nucleophilic substitution, a monomolecular elimination (E-1 mechanism) also occurs; if the time of reaction is long enough (for example 12 hr.; see Table 1), then water or methanol is completely eliminated and 6-dehydro-oestrone is the single reaction product.

The isolation of 6 α -hydroxyoestrone confirms the previous statement of Marrian and his colleagues that in the urine of pregnant women there may be a 6-hydroxyoestrone (Marrian & Sneddon, 1960) referred to as Kober chromogen KC-6B (Loke *et al.* 1959).

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