Studies on the Metabolism of Steroids in the Foetus

BIOSYNTHESIS OF 6 α -HYDROXYTESTOSTERONE IN THE HUMAN FOETAL LIVER

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After incubation of testosterone with 105 000g microsomes of human foetal liver, 6 α -hydroxytestosterone was isolated and identified by t.l.c. and g.l.c.-mass spectrometry. This is the first example of 6 α -hydroxylation of C₁₉ steroids in the human liver, and the finding is discussed in relation to earlier reports of 6-oxygenated C₁₉ and C₁₈ steroids in pregnant women.

Schubert, Wehrberger & Frankenberg (1964) first isolated and identified 6 α -hydroxytestosterone as a metabolite in human urine after intravenous administration of testosterone to adult women. The tissue site of 6 α -hydroxylation was unknown, and it could not be excluded that the metabolite had arisen by epimerization of 6 β -hydroxytestosterone.

Recently we demonstrated that testosterone is hydroxylated in positions 1 β , 2 β , 6 β and 18 after incubations with microsomes from livers of mid-pregnancy fetuses (Gustafsson & Lisboa, 1968; Lisboa & Gustafsson, 1968, 1969a). Minor amounts of two other unknown C₁₉O₃ steroids with a 3-oxo- Δ^4 structure were also found in these incubation studies. One of them has now been isolated and characterized as 6 α -hydroxytestosterone.

MATERIALS AND METHODS

Steroids. Testosterone (17 β -hydroxyandrost-4-en-3-one), obtained by courtesy of Dr J. Ufer, Schering A.-G., Berlin, Germany, was recrystallized twice before use. 6 β -Hydroxytestosterone and 6 α -hydroxyandrostenedione (6 α -hydroxyandrost-4-en-3-one) were kindly supplied by Dr L. Reineke and Professor M. Ehrenstein respectively. 6 α -Hydroxytestosterone was biosynthesized by incubation of 6 α -hydroxyandrostenedione with 105 000g liver microsomes of adult male rats at pH 6.8 (J.-Å. Gustafsson & B. P. Lisboa, unpublished work).

Clinical material. Foetal liver tissue was obtained from three male fetuses at mid-pregnancy from patients who were admitted to the Karolinska Sjukhuset for legal abortion for medico-social reasons.

Thin-layer chromatography. Multiple one-dimensional t.l.c. (cf. Lisboa, 1969) was carried out on precoated silica gel GF₂₅₄ layers (E. Merck A.-G., Darmstadt, Germany; no. 5717, preparative 2mm. layers; no. 5715, analytical 250 μ m. layers). The following solvent systems were used: S-1, ethyl acetate-*n*-hexane-acetic acid (15:4:1, by vol.) (two developments); S-2, chloroform-ethanol (9:1, v/v) (two developments); S-3, ethyl acetate-cyclohexane (3:2, v/v) (four developments). The 3-oxo- Δ^4 steroids were

located by observation of the plates under a short-wave u.v. lamp 254nm.). The compounds were eluted from the silica gel with ethanol. For this purpose successive layers of quartz, Celite and silica gel scraped from the t.l.c. plate were packed in a column having a bottom of sintered glass. All eluates were submitted to g.l.c.-mass-spectrometry analysis.

G.l.c.-mass spectrometry. The LKB 9000 gas chromatograph-mass spectrometer (LKB-Produkter AB, Stockholm, Sweden) was used. The steroids were analysed both as trimethylsilyl ('silyl') ethers (Makita & Wells, 1963) and as *O*-methyloxime trimethylsilyl ('oxime-silyl') derivatives (Gardiner & Horning, 1966) by using two different stationary phases, 3% (w/v) QF-1 and 1% SE-30 on 80-100-mesh Gas Chrom Q. Coiled glass columns (2-3m. $\times$ 3.5mm.) were used. Chromatographic conditions were as follows: column temperature, 238° (SE-30) and 220° (QF-1); flash-heater temperature, 260°; molecule-separator and ion-source temperatures, 260° and 290° respectively; helium flow rate, 35ml./min. Retention times were determined relative to that of 5 α -cholestane (*t*_R). The energy of the bombarding electrons was 22.5ev and the ionizing current was 60 μ A.

Preparation of microsomes and conditions of incubation. The foetal liver tissue obtained immediately after operation was weighed, cut into small pieces and homogenized in 0.25M-sucrose at 4° with a loose-fitting Teflon pestle. The 20% (w/v) liver homogenate was submitted to differential centrifugation in a Spinco ultracentrifuge (L50). After centrifugation for 10min. at 10 000g, the supernatant obtained was centrifuged at 105 000g for 60min. The microsomal pellet was resuspended in ice-cold 0.25M-sucrose and centrifuged again at the same speed for 1hr. Finally the microsomes were resuspended in a sucrose solution to give a concentration equivalent to 0.5g. of fresh tissue/ml. Protein determinations carried out by the method of Lowry, Rosebrough, Farr & Randall (1951) showed a protein concentration between 5.0 and 7.3mg./ml., with crystalline freeze-dried bovine serum albumin as a standard.

Liver microsome preparations corresponding to 0.5g. of tissue/ml. were incubated with steroids (steroid/tissue ratio 1:1000; testosterone, 5mg./5g. of tissue; 6 β -hydroxytestosterone, 1.1mg./1.1g. of tissue). An NADPH-regenerating system was used and the incubation was carried out

at 37° for 60 min. The incubation medium had the following composition: MgCl_2 , 5 mM; MnCl_2 , 5 μM ; nicotinamide, 5 mM; NADP^+ , 1 mM; isocitrate, 5 mM; isocitrate dehydrogenase, 0.01% (w/v); tris buffer, pH 7.5, 75 mM. The final volumes were 20 ml. and 4 ml. for the incubations with testosterone and 6 β -hydroxytestosterone respectively. The incubations were stopped by addition of 20 ml. of ether-chloroform (3:1, v/v). The incubation mixture was extracted four times with the same solvent mixture, and the extracts were combined, filtered, evaporated under reduced pressure and redissolved in a small volume of ethanol.

RESULTS

Incubation with testosterone. Three sets of incubations were carried out. Each of the three extracts was chromatographed in solvent system S-1. The zone between 7 and 9.5 cm., which contained four 3-oxo- Δ^4 C_{19}O_3 steroids, was then

Table 1. Relative retention times (5 α -cholestane = 1.00) of the silyl ethers and oxime-silyl derivatives of the isolated steroid and of 6 α -hydroxytestosterone on 1% SE-30 and 3% QF-1

Compound	Relative retention time		
	SE-30		QF-1
	Silyl ether	Oxime-silyl derivative	
Isolated compound	1.06	0.92:1.00	1.26
6 α -Hydroxytestosterone	1.06	0.94:1.00	1.29

rechromatographed in solvent system S-2. An unknown C_{19}O_3 steroid showing u.v. absorption was isolated from the zone between 9.0 and 10.5 cm. It was separated from the more polar 1 β -hydroxytestosterone and the less polar 6 β - and 2 β -hydroxytestosterone, but it was still somewhat contaminated with 6 β - and 2 β -hydroxytestosterone. After further purification in solvent system S-3 it was analysed by g.l.c.-mass spectrometry. The t_R values of the silyl ether and the oxime-silyl derivative on SE-30 and of the oxime-silyl derivative on QF-1 were the same as those of the derivatives of 6 α -hydroxytestosterone (see Table 1). The silyl ethers of the isolated compound and of 6 α -hydroxytestosterone gave mass spectra that were identical within the experimental variation (Fig. 1). The oxime-silyl derivatives of the isolated compound and 6 α -hydroxytestosterone both produced two peaks on the SE-30 column; this is due to a *syn-anti*-isomerism of the oxime-silyl derivatives (Horning, Moss & Horning, 1968; J.-Å. Gustafsson & B. P. Lisboa, unpublished work). Fig. 2 shows the high-mass end of the spectra recorded for the oxime-silyl derivatives of 6 α -hydroxytestosterone and the unknown compound when run on QF-1; the spectra are identical. The low-mass end of the spectrum of the isolated steroid contained many peaks due to the presence of non-steroid material.

Incubation with 6 β -hydroxytestosterone. The extract from this incubation was run in solvent system S-2. The silica gel was scraped off in several zones: I (0.5–5.0 cm. from the starting line), II (5.0–7.0 cm.), III (7.0–9.0 cm.), IV (9.0–10.5 cm.,

Fig. 1. Mass spectrum of the silyl ethers of 6 α -hydroxytestosterone (a) and the isolated compound (b).

Fig. 2. High-mass end of the mass spectra of the oxime-silyl derivatives of 6 α -hydroxytestosterone (a) and the isolated compound (b).

i.e. mobility of 6 α -hydroxytestosterone) and V (10.5–12.0 cm., containing most of the incubated steroid). 6 α -Hydroxytestosterone was not detected in any of these zones.

DISCUSSION

The isolation of 6 α -hydroxytestosterone after incubation of testosterone with 105 000g microsomes obtained from human foetal liver indicates the presence in this tissue of a 6 α -hydroxylase active for 3-oxo- Δ^4 C₁₉ steroids. Under similar conditions progesterone (pregn-4-ene-3,20-dione) is metabolized to 6 α -hydroxyprogesterone (Lisboa & Gustafsson, 1969b). The ability of liver tissue to hydroxylate 3-oxo- Δ^4 steroids at position 6 α is not limited to the human foetus; liver microsomes from adult male rats carry out 6 α -hydroxylation of testosterone and androstenedione (J.-Å. Gustafsson & B. P. Lisboa, unpublished work) as well as of progesterone (Lisboa & Gustafsson, 1969c).

Several steroids with a 6 α -hydroxyl group have been isolated from human urine. Lieberman, Dobriner, Hill, Fieser & Rhoads (1948) and Lieberman, Fukushima & Dobriner (1950) isolated 3 α ,6 α -dihydroxy-5 α -pregnan-20-one and 3 α ,6 α -dihydroxy-5 β -pregnan-20-one from pregnancy urine, and Frantz, Katz & Jailer (1960, 1961) showed the presence of 6 α -hydroxycortisol (6 α ,11 β ,17 α ,21-tetrahydroxypregn-4-ene-3,20-dione) in urine from pregnant, non-pregnant and diseased humans. Further, Harkness & Fotherby (1961) identified labelled 3 α ,6 α -dihydroxy-5 β -pregnan-20-one in the urine of an ovariectomized adrenalectomized woman after administration of [4-¹⁴C]progesterone. Finally, after intravenous administration of testosterone phosphate to four women, Schubert, Wehrberger &

Scheme 1. Proposed pathway for the biosynthesis of 6-oxygenated phenolic steroids during pregnancy. Abbreviations: T, testosterone; A⁴, androstenedione; E₁, oestrone; E₂, oestradiol.

Frankenberg (1964) isolated 0.1% of the metabolites as 6 α -hydroxytestosterone.

Although the adult liver obviously can be the site of 6 α -hydroxylation, our results indicate that during pregnancy 6 α -hydroxylation of 3-oxo- Δ^4 steroids can also occur in the foetal liver. 6 α -Hydroxylated oestrogens in urine from pregnant subjects (cf. Knuppen & Breuer, 1968) have previously been thought to arise principally via 6 α -hydroxylation of oestrogen precursors in the foetal liver (Breuer, Knuppen, Ortlepp, Pangels & Puck, 1960) and in the placenta (Alonso & Troen, 1964, 1966). A third pathway may now be considered: 6 α -hydroxylation of neutral C₁₉ steroids in the foetal liver with subsequent aromatization, possibly in the placenta. Thus Breuer, Knuppen & Pangels (1961) have shown that microsome preparations from human placenta can aromatize 6 α -hydroxylated 3-oxo- Δ^4 C₁₉ steroids. A summary of these pathways is given in Scheme 1.

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