16 β -Hydroxylation of Dehydroepiandrosterone Sulphate by Homogenates of Human Foetal Liver

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The chemical syntheses of the 3-sulphates of 16 α -acetoxy-, 16 α -hydroxy- and 16 β -hydroxy-dehydroepiandrosterone as their triethylammonium salts are described preparatory to studies of the metabolism of [7 α -³H]dehydroepiandrosterone sulphate by homogenates of human foetal liver. Five main radioactive products of the incubation were separated by partition chromatography on a Celite column. Two were identified as 16 α - and 16 β -hydroxydehydroepiandrosterone 3-sulphates by crystallization to constant specific radioactivity before and after solvolysis. The yields of the conversion to the two epimers were 24.4% (16 α) and 1.8% (16 β).

The human foetus and placenta exhibit striking differences in steroid metabolism. Foetal tissue, especially adrenal cortex, possesses considerable sulphotransferase activity (Wengle, 1966), whereas the placenta has an extensive capacity for the hydrolysis of 3 β -sulphates of 5-ene steroids (Bolté *et al.*, 1964; Lamb *et al.*, 1967). In consequence, most steroids circulate in the foetal compartment as ester sulphates and some are important precursors of hormonal steroids in the 'foetoplacental unit' (Diczfalusy, 1967).

16 α -Hydroxydehydroepiandrosterone 3-sulphate is quantitatively the most significant steroid in the foetal circulation (Huhtaniemi & Vihko, 1970; Laatikainen & Peltonen, 1975) and its 16 β -epimer occurs as the disulphate ester in human neonatal urine (Shackleton *et al.*, 1968, 1971), in cord plasma (Mitchell & Shackleton, 1969), in amniotic fluid (Mitchell & Shackleton, 1969; Luukkainen *et al.*, 1970) and in adult bile (Laatikainen, 1970). This compound is also found as its monosulphate in adult urine at 10–15% of the concentration of its 16 α -epimer (Jänne & Vihko, 1969), and high urinary excretions of the steroid have recently been described in patients with low renin hypertension by Liddle & Sennett (1975) who reported that 16 β -hydroxy-dehydroepiandrosterone exhibits mineralocorticoid activity about equal to that of 11-deoxycorticosterone in the adrenalectomized rat.

The mechanism(s) whereby 16 α - and 16 β -hydroxylated steroids are formed are not clear and the aim of this study was to demonstrate the extent of formation of such derivatives of dehydroepiandrosterone

sulphate by preparations of human foetal liver, before investigation of the enzyme(s) involved.

The capacity of human foetal liver to hydroxylate oestrogens at C-16 has been known for some time (Engel *et al.*, 1962; Slaunwhite *et al.*, 1965), and Ingelman-Sundberg *et al.* (1975) have described some properties of hydroxylase systems in microsomal preparations of human foetal liver, but they did not find 16 β -hydroxydehydroepiandrosterone or its 3-sulphate in their experiments.

Experimental

Materials

Water and all organic solvents were redistilled before use. NADPH and 16 α ,17 α -epoxy-3 β -hydroxy-pregn-5-en-20-one were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Dehydroepiandrosterone and dehydroepiandrosterone acetate were from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Silica gel G (Macherey, Nagel and Co., Düren, Germany) was obtained from Camlab, Cambridge, U.K., and Celite 545 was from Johns-Manville Corp., New York, NY, U.S.A., and was washed before use (Engel *et al.*, 1961). DEAE-cellulose (Whatman DE 11, Cl⁻ form) was from H. Reeve Angel and Co., London E.C.4, U.K., and used in accordance with the manufacturers' recommendations.

Tissue

Human foetal liver was obtained from legal terminations of pregnancy. Livers were removed within 30 min of operation and stored in air-tight containers at –20°C for periods of up to 3 months. Liver no. 1 was from a female foetus and no. 2 from a male, both aged 20 weeks.

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Syntheses

16 β -Hydroxydehydroepiandrosterone was prepared by the method described by Wynne & Renwick (1972) and 16 α -acetoxydehydroepiandrosterone was synthesized in three steps from 16 α ,17 α -epoxy-3 β -hydroxypregn-5-en-20-one (Ahkrem *et al.*, 1970).

Dehydroepiandrosterone sulphate was prepared by treating 1.5 mmol of H₂SO₄ with 1.0 mmol of dehydroepiandrosterone by the method described by Mumma *et al.* (1969), modified as follows: the chromatography on DEAE-cellulose was performed with 200 ml of 0.6 M-triethylammonium sulphate instead of methanolic NH₃. The effluent was extracted twice with 375 ml of chloroform, the combined extracts were cooled to -20°C, filtered and evaporated to dryness under reduced pressure. The residue was recrystallized from acetone/methanol/hexane to give dehydroepiandrosterone sulphate as the triethylammonium salt, m.p. 219–222°C. The reported m.p. is 220–222°C (Dusza *et al.*, 1968).

16 α -Acetoxydehydroepiandrosterone sulphate was prepared in 50% yield by the same method. The triethylammonium salt was recrystallized from dichloromethane/ether and methanol/diethyl ether as white crystals with m.p. 206–208°C (Kofler) and $[\alpha]_D^{21} + 0.5 \pm 0.5^\circ$ (c 0.1 in chloroform) (Found: C, 61.0; H, 8.4; N, 2.8; S, 5.6. Calc. for C₂₇H₄₅NO₇S: C, 61.5; H, 8.6; N, 2.7; S, 6.1%). The i.r. $\nu_{\max}$ were 3480–3400, 2930, 2675, 1755, 1740 and 1250–1230 cm⁻¹.

The triethylammonium salt of 16 α -hydroxydehydroepiandrosterone 3-sulphate was prepared by leaving a solution of the acetate ester in triethylamine and methanol (1:4, v/v) at room temperature for 7 days. The residue was recrystallized twice from acetone to give white crystals with m.p. 157–165°C (Kofler) and $[\alpha]_D^{21} + 7 \pm 0.5^\circ$ (c 0.1 in chloroform) (Found: C, 62.0; H, 8.8; N, 3.1; S, 6.6. Calc. for C₂₅H₄₃NO₆S: C, 61.8; H, 8.9; N, 2.9; S, 6.6%). The i.r. $\nu_{\max}$ were 3460–3380, 2935, 2675, 1740, 1250–1230, and 985 cm⁻¹.

16 β -Hydroxydehydroepiandrosterone 3-sulphate was similarly prepared by reacting 1.1 mmol of H₂SO₄ with 1.0 mmol of unconjugated steroid. The triethylammonium salt was recrystallized from acetone and methanol/diethyl ether to give white crystals with m.p. 158–160°C (Kofler) and $[\alpha]_D^{21} + 4 \pm 0.5^\circ$ (c 0.1 in chloroform) (Found: C, 61.5; H, 8.6; N, 2.8; S, 6.2. Calc. for C₂₅H₄₃NO₆S: C, 61.8; H, 8.9; N, 2.9; S, 6.6%). The i.r. $\nu_{\max}$ were 3450–3360, 2930, 2675, 1740, 1245–1235 cm⁻¹.

The pyridinium salt of androst-5-ene-3 β ,17 β -diol 3-sulphate was prepared by the method described by McKenna & Rippon (1965).

Radioactive substrate

[7 α -³H]Dehydroepiandrosterone sulphate (NH₄⁺ salt dissolved in ethanol; 0.25 mCi/9.25 μ g) was from

New England Nuclear Corp., U.S.A. This was converted into the triethylammonium salt (Mickan *et al.*, 1969), mixed with non-radioactive dehydroepiandrosterone sulphate (triethylammonium salt), and recrystallized first from acetone/methanol/hexane and then from dichloromethane/ether. The crystals were dried under reduced pressure over P₂O₅ and dissolved in ethanol to give a stock solution of 1.50 mg/ml. Determination of radioactivity showed that the specific radioactivity of the salt was 0.235 μ Ci/ μ mol and that of the stock solution was 16.6 μ Ci/ml. Immediately before use in incubations, samples of stock solution were examined by t.l.c. in system 1 (see below) and scanned for radioactivity to ensure that no trace of unconjugated steroid was present.

Measurement of 16 α - and 16 β -hydroxydehydroepiandrosterone 3-sulphates

The method was based on that described by Oertel & Eik-Nes (1959) for the determination of steroids possessing a 3 β -hydroxy-5-ene structure. Conc. H₂SO₄ (2 vol.) were mixed with 1 vol. of 95% (v/v) ethanol in an ice bath. The ethanol was freshly distilled under reduced pressure over Ag₂O. Freshly prepared reagent (0.5 ml) was added to a dry residue of steroid or steroid sulphate (0.5–5 μ g). The mixtures were left at room temperature (20°C) for 90 min (Bongiovanni, 1965), and E₄₀₅ and E₆₀₅ determined with a reagent blank. Standard curves were constructed from known amounts of authentic material. 16 α -Hydroxydehydroepiandrosterone 3-sulphate gave a maximum extinction at 610 nm (ϵ 1.52 $\times 10^4$ litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹), whereas the 16 β epimer was measured at 405 nm (ϵ 1.87 $\times 10^4$ litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹).

Incubation procedure

Foetal tissue (1 g) was gently disrupted in a Potter-Elvehjem homogenizer with 10 ml of Na₂HPO₄/NaH₂PO₄ buffer (50 mM, pH 7.4). A 4 ml sample (equivalent to 400 mg wet wt. of tissue) was added to a flask containing 3.1 mg of NADPH in 0.3 ml of buffer and 0.1 ml (1.66 μ Ci) of [7 α -³H]dehydroepiandrosterone sulphate solution dissolved in ethanol. The flask was incubated at 37°C for 1 h in a mechanical water bath; 4 ml of 1.5 M-triethylammonium sulphate (Mickan *et al.*, 1969) was added and the mixture extracted three times with 16 ml volumes of dichloromethane. The combined extracts were cooled to -20°C, filtered through glass wool and evaporated to dryness under reduced pressure.

Control incubations

Portions of liver homogenate were boiled and incubated with [7 α -³H]dehydroepiandrosterone sulphate and NADPH under the conditions described

above. After extraction the dry residue was chromatographed in system 2 and scanned for radioactivity.

Solvolysis

Crude extracts from some incubations and some combined fractions were solvolysed by the method of Vihko (1966).

Chromatography

Ascending t.l.c. was carried out on glass plates coated with 0.25 mm of silica gel G in the following solvent systems: (1) upper layer [benzene/acetone/water (2:1:2, by vol.)]/methanol (7:3, v/v) (Joseph *et al.*, 1966); (2) iso-octane/chloroform/methanol/triethylamine (6:12:2:1, by vol.); (3) benzene/ethanol (19:1, v/v) (Shackleton & Mitchell, 1967).

Free steroids obtained by solvolysis were examined by t.l.c. by using three developments in system 3. Reference compounds were located after spraying with 10% (w/v) phosphomolybdic acid dissolved in methanol and by heating to 100°C.

The partition system described by Engel *et al.* (1961) was adapted for use with the solvent system

chloroform/iso-octane/butanone/methanol/0.15M-triethylammonium sulphate (5:5:4:1:5, by vol.). A 35 g column (1.2 cm $\times$ 75 cm) was packed with Celite mixed with aqueous phase (2:1, w/v) under a head of mobile phase.

Both 16 α - and 16 β -hydroxydehydroepiandrosterone 3-sulphate (100 μ g of each) dissolved in ethanol were added to each extract of an incubation mixture and dried under reduced pressure. The residue was dissolved in 0.5 ml of stationary phase, mixed with 1 g of Celite and packed on to the top of the column. Elution was carried out with mobile phase at a rate of 10 ml/h and fractions were collected by time on an LKB Radi Rac. When the total effluent measured 420 ml, the mobile phase was changed to the lower layer of the system chloroform/methanol/0.15M-triethylammonium sulphate (9:1:5, by vol.). A further 200 ml of effluent was collected. Of each fraction 0.5–2.0 ml was removed for analysis.

Results

When [7 α - ^{3} H]dehydroepiandrosterone sulphate was incubated with homogenates of human foetal

Fig. 1. Partition chromatography of steroid sulphates from an incubation of human foetal liver homogenate with [7 α - ^{3} H]dehydroepiandrosterone sulphate

A homogenate of foetal liver no. 2 was incubated with [7 α - ^{3} H]dehydroepiandrosterone sulphate. The extracted radioactive metabolites and added carrier 16 α - and 16 β -hydroxydehydroepiandrosterone 3-sulphates were chromatographed as triethylammonium salts on a column (35 g; 1.2 cm $\times$ 76 cm) of Celite 545 and eluted at a flow rate of 10 ml/h. Fractions were collected and analysed for radioactivity and Oertel-Eik-Nes chromogens (Oertel & Eik-Nes, 1959). Δ , ^3H radioactivity (d.p.s./ml); $\circ$, 16 α -hydroxydehydroepiandrosterone 3-sulphate ($\mu\text{g/ml}$); $\bullet$, 16 β -hydroxydehydroepiandrosterone 3-sulphate ($\mu\text{g/ml}$). The solvents used were: (a) iso-octane/chloroform/butanone/methanol/0.15M-triethylammonium sulphate (5:5:4:1:5, by vol.); (b) chloroform/methanol/0.15M-triethylammonium sulphate (9:1:5, by vol.).

Fig. 2. Specific radioactivity of some steroid sulphates from an incubation of foetal liver with $[7\alpha\text{-}^3\text{H}]$ dehydroepiandrosterone sulphate

A portion of the data from Fig. 1 [peaks (II) and (III)] replotted as specific radioactivities, i.e. (○) 16α -hydroxydehydroepiandrosterone 3-sulphate and (●) 16β -hydroxydehydroepiandrosterone 3-sulphate (^3H d.p.s./ μg). Between the 16α - and 16β -epimers it is apparent there is radioactivity that is not associated with carrier steroid. This 'hidden peak' was subsequently tentatively identified as androst-5-ene- 3β , 17β -diol 3-sulphate (Table 1).

liver and its metabolic products were subjected to t.l.c. and scanned for radioactivity, most of the ^3H was associated with a compound of greater polarity than dehydroepiandrosterone sulphate. In contrast, extracts from control incubations give a single peak of radioactivity with the same chromatographic mobility as authentic dehydroepiandrosterone sulphate. Column chromatography of steroids from incubations with foetal livers no. 1 and 2 revealed almost identical profiles of radioactivity in the effluent, where five major peaks were apparent (Fig. 1).

Peak (I) (Fig. 1) was shown by t.l.c. to contain a single radioactive spot with the same R_F as the reference compound dehydroepiandrosterone sulphate. No unconjugated radioactive steroids were detected at the solvent front. Peak (II) (Fig. 1) was associated with the carrier compound, 16α -hydroxydehydroepiandrosterone 3-sulphate. A sample of the effluent containing this peak was crystallized with the triethylammonium salt of the carrier; the remainder was solvolysed and after the addition of 16α -hydroxydehydroepiandrosterone, both were recrystallized to constant specific radioactivity. Peak (III) and authentic 16β -hydroxydehydroepiandrosterone 3-sulphate were coincident, and the triethylammonium salt of the 3-sulphate and unconjugated 16β -hydroxydehydroepiandrosterone were employed as carriers in crystallizations to constant specific radioactivity. The results show that the original sample was not radiochemically pure. When the specific radioactivities of the 16α - and 16β -hydroxydehydroepiandrosterone 3-sulphates were examined more closely and plotted against effluent volume (Fig. 2) it was apparent that another metabolite lay between peaks (II) and (III) (Fig. 1). By using this portion of the effluent an attempt was made to identify the compound by crystallization to constant specific

Table 1. Specific radioactivities of presumptive androstenediol 3-sulphate isolated from an incubation with foetal liver no. 2 and $[7\alpha\text{-}^3\text{H}]$ dehydroepiandrosterone sulphate

The compound was isolated by column chromatography as the triethylammonium salt and was found as a contaminant of the combined fractions containing 16β -hydroxydehydroepiandrosterone 3-sulphate. It was subjected to successive crystallizations with authentic carrier from a series of solvent pairs.

Crystallization	Solvent pair	Specific radioactivity of androstenediol 3-sulphate (pyridinium salt; 166000 d.p.m.) (d.p.m./mg)	
		Crystals	Mother liquor
1	Methanol/diethyl ether	11070	17830
2	Acetone/methanol	5240	15110
3	Chloroform/diethyl ether	4521	7309
4	Methanol/diethyl ether	3834	5069
5	Acetone/methanol	3871	3677

Fig. 3. Radiochromatogram of solvolysed steroid sulphates from an incubation of foetal liver with $[7\alpha\text{-}^3\text{H}]$ dehydroepiandrosterone sulphate

A sample of foetal liver no. 1 was incubated with $[7\alpha\text{-}^3\text{H}]$ dehydroepiandrosterone sulphate as described in the text. One-quarter of the dichloromethane extract was solvolysed and the free steroids were examined by t.l.c. on silica gel by using three developments in benzene/ethanol (19:1, v/v). The reference compounds used were: DHA, dehydroepiandrosterone; 16α - and 16β -OHDHA, 16α - and 16β -hydroxydehydroepiandrosterone; $3\beta,16\alpha,17\beta$ -A-5-triol, androst-5-ene- $3\beta,16\alpha,17\beta$ -triol.

Table 2. Recoveries of radioactivity after incubation of $[7\alpha\text{-}^3\text{H}]$ dehydroepiandrosterone sulphate with homogenates of human foetal liver

Human foetal liver (400mg) was homogenized in a Potter-Elvehjem homogenizer with 10vol. of 50mM-sodium phosphate buffer, pH7.4. The homogenate (4ml) was incubated at 37°C for 1 h in a conical flask containing 3.1 mg of NADPH in 0.3 ml of buffer and 0.1 ml (1.66 μCi) of $[7\alpha\text{-}^3\text{H}]$ dehydroepiandrosterone sulphate dissolved in ethanol. The yields of the steroid sulphates were calculated from data obtained by crystallization to constant specific radioactivity and represent minimum values uncorrected for procedural losses.

Incubation	Radioactivity (% of that in original substrate)				
	Dichloromethane extract	Column effluent	16α -Hydroxydehydroepiandrosterone 3-sulphate	16β -Hydroxydehydroepiandrosterone 3-sulphate	Androstenediol 3-sulphate
Liver no. 1	82.3	58.3	24.4	1.8	
Liver no. 1 (boiled)	81.8				
Liver no. 2	86.0	70.2	38.0*	4.0*	5.8
Liver no. 2 (boiled)	88.2				

* Calculated from analyses of effluent peaks (Fig. 1).

radioactivity with the pyridinium salt of androst-5-ene-3 β ,17 β -diol 3-sulphate (Table 1).

Peaks (IV), (V) and (VI) (Fig. 1) were not identified, although they represent 1.8, 14.9 and 0.8% respectively of the total radioactivity in the column effluent. Solvolysis of a dichloromethane extract of an incubation with liver no. 1, and subsequent radiochromatography, showed that a major metabolite had the same chromatographic mobility as androst-5-ene-3 β ,16 α ,17 β -triol (Fig. 3). Procedural recoveries of radioactivity and yields of isolated steroid sulphates are presented in Table 2.

Discussion

Evidence for the formation of 16 α - and 16 β -hydroxydehydroepiandrosterone 3-sulphate *in vitro* by homogenates of foetal liver is adduced from: (a) similar chromatographic behaviour of presumptive metabolites before and after solvolysis in the presence of reference compounds; (b) coincidence of the triethylammonium salts of those metabolites with authentic steroids on column chromatography; (c) crystallization to constant specific radioactivity, whereby the criteria for radiochemical purity proposed by Axelrod *et al.* (1965) are fulfilled, i.e. the specific radioactivities of crystals obtained in the last three crystallizations are within $\pm 5\%$ of their mean. When presumptive 16 α -hydroxydehydroepiandrosterone 3-sulphate was solvolysed and mixed with authentic unconjugated carrier, the series of four crystallizations only just failed to meet the same criteria of radiochemical purity if an aberrant result for the third crystallization was omitted. We can offer no satisfactory explanation for this result.

The results relating to the 16 β -epimer were weakened by the presence of extraneous radioactivity during crystallization to constant specific radioactivity. This impurity was a radiometabolite (Fig. 2) located on the polar side of 16 α -hydroxydehydroepiandrosterone 3-sulphate [Fig. 1, Peak (II)]. Complete resolution of peaks (II) and (III) was not achieved by column chromatography of the steroid reactants from the first incubation, so that this unsuspected metabolite was included with presumptive 16 β -hydroxydehydroepiandrosterone 3-sulphate. Despite this, six crystallizations of the monosulphate met the requirements for radiochemical purity. Repeated crystallizations of the unconjugated steroid gave indications of reaching constant specific radioactivity, but solvolysis of 16 β -hydroxydehydroepiandrosterone 3-sulphate resulted in a gross increase of radioactive contamination, which was almost certainly due to acid-induced isomerization. Additional evidence of identity was obtained from Fig. 2, where the specific radioactivities of six successive column fractions are constant and those fractions represent more than 90% of peak (III) (Fig. 1).

Androst-5-ene-3 β ,17 β -diol 3-sulphate (Table 1) and androst-5-ene-3 β ,16 α ,17 β -triol (Fig. 3) were only tentatively identified.

Slaunwhite *et al.* (1965) claimed a 12% conversion of unconjugated dehydroepiandrosterone into androst-5-ene-3 β ,16 β ,17 β -triol in similar studies with human foetal liver, but the triol, isolated as an acetone, was not recrystallized to constant specific radioactivity, because of the lack of authentic compound. The results of this investigation were also complicated by the use of a procedure whereby all ketones were reduced with KBH₄ before extraction and separation of steroid products from incubation mixtures. The isolated triol may have arisen from 3 β ,17 β -dihydroxyandrost-5-en-16-one, or it may have been androst-5-ene-3 β ,16 α ,17 α -triol, which also forms an acetone.

Ingelman-Sundberg *et al.* (1975) incubated dehydroepiandrosterone sulphate with the microsomal fraction of human foetal liver and reported two products, 16 α -hydroxydehydroepiandrosterone 3-sulphate and 3 β ,17 β -dihydroxyandrost-5-en-16-one 3-sulphate in a ratio of about 1:2. The latter compound was thought to have been derived from the former, but the 17 β -hydroxy-16-oxo ketol was not identified. The g.l.c. system employed in this study is not recognized as being capable of separating 16 β -hydroxydehydroepiandrosterone from 3 β ,17 β -dihydroxyandrost-5-en-16-one (Shackleton *et al.*, 1968; Jänne & Vihko, 1969; K. N. Wynne, unpublished work), so the presence of the 16 β -hydroxy compound is not precluded. Support for this possibility may be deduced from their finding that about equal quantities of 16 α - and 16 β -hydroxy-oestrone were produced when oestrone sulphate was incubated under similar conditions to those employed with dehydroepiandrosterone sulphate.

Although the above explanation seems the more plausible for the absence of the 16 β -hydroxy compound in the Swedish experiments (Ingelman-Sundberg *et al.*, 1975), our experimental conditions were not directly comparable in that they used microsomal preparations of foetal liver, whereas we did not fractionate that tissue. If the '16 β -hydroxylase' is not associated with the microsomal fraction it is possible that such an enzyme could be associated with another organelle, and the mitochondrion offers the most likely alternative site because steroid hydroxylases so far described are membrane bound. On the other hand, 16 β -hydroxy compounds could be formed from 16-oxo substrates by soluble specific oxidoreductases, or by a 16-hydroxy steroid epimerase such as that which occurs in human placenta, liver and intestine (Dahm *et al.*, 1968).

It has been suggested (Ingelman-Sundberg *et al.*, 1975) that the purpose of the steroid sulphate-hydroxylating system in human foetal liver is to

provide a detoxification mechanism for biologically active steroids that cannot be excreted. This teleological argument is not convincing when one considers that 16-hydroxylation is not unique to the foetal compartment; it is also quantitatively important in the adult (Baulieu *et al.*, 1965; Laatikainen, 1970; Gurrpide *et al.*, 1973; Seth & Pennington, 1973) and perhaps the demonstration of the mineralocorticoid action of 16 β -hydroxydehydroepiandrosterone (Liddle & Sennett, 1975) heralds reassessment of its role in human metabolism.

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