acid are found in the range 2.5–2.7 (Bovey & Tiers, 1959) and the olefinic protons of similar compounds to (I) are found around 4.2 (Ettlinger & Kennedy, 1956).

The α -proton τ value of 6.13 was slightly higher than that characteristic of α -protons in straight-chain amino acids (5.2–5.8; Bovey & Tiers, 1959), and that of the cyclopropane protons is considerably lower than the value found in cyclopropane itself (9.78; Jackman, 1959). However, these differences are not surprising as the cyclopropane ring would be expected to modify the chemical shift of the

protons near to it, and similarly the olefinic group would be expected to change the electron density in the cyclopropane ring itself.

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The 16 α -Hydroxylation of Dehydroepiandrosterone (3 β -Hydroxy-androst-5-en-17-one) by Rat-Liver Slices

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The isolation of 16 α -hydroxydehydroepiandrosterone (3 β ,16 α -dihydroxyandrost-5-en-17-one) from human urine was first reported by Fotherby, Colás, Atherden & Marrian (1956, 1957). They also obtained chromatographic evidence for its presence in the urine of a 45-year-old woman (who had undergone bilateral ovariectomy and bilateral adrenalectomy as treatment for a carcinoma of the breast) after she received 100 mg. of dehydroepiandrosterone intramuscularly. They suggested that 16 α -hydroxydehydroepiandrosterone was an intermediate in the transformation of dehydroepiandrosterone into androst-5-ene-3 β ,16 α ,17 β -triol.

Several other 16 α -hydroxy steroids have been isolated from human urine. Besides the neutral steroids listed by Fotherby (1959) and the three phenolic steroids (Breuer, 1960), Okada, Fukushima & Gallagher (1959) have reported the isolation of 3 β ,16 α -dihydroxyandrost-5-ene-7,17-dione and 3 β ,7 α ,16 α -trihydroxyandrost-5-en-17-one from the urine of a patient with adrenal carcinoma; Neher, Meystre & Wettstein (1959) found 3 α ,16 α -dihydroxy-5 α -pregnan-20-one and 3 α ,16 α -dihydroxy-5 β -pregnan-20-one in the urine of patients showing the adrenogenital syndrome. Brooks & Klyne (1957) identified 5 α -androstane-3 β ,16 α -diol from pregnant mare's urine, whereas 3 β ,16 α -dihydroxy-5 α -pregnan-20-one has been isolated from hog adrenals (Neher, Desaulles, Vischer, Wieland & Wettstein, 1958), and 16 α -hydroxytestosterone (16 α ,17 β -dihydroxyandrost-4-en-3-one) from bull testes (Neher & Wettstein, 1960). Two of the

pregnane derivatives (Neher *et al.* 1958, 1959), under certain biological conditions, appear to increase excretion of sodium, paralleling some of the effects observed with a number of synthetic 16 α -hydroxy steroids (Bernstein, 1958). On the other hand, the biological significance of 16 α -hydroxylation is further demonstrated by the different effectiveness of oestriol and oestradiol in stimulating the various parts of the female genital tract (Merrill, 1958).

The work done *in vitro* on the reactions leading to the appearance of a 16 α -hydroxyl group in the molecule of phenolic steroid hormones is extensive (cf. review, Breuer, 1960). In most cases, liver slices of different species (man, rat, dog) have been used, though human ovary is also effective. In contrast, there are few reports of 16 α -hydroxylation of neutral steroids *in vitro*: Schneider & Mason (1948) incubated sodium dehydroepiandrosterone hemisuccinate with rabbit-liver slices and obtained androst-5-ene-3 β ,16 α ,17 β -triol; Axelrod, Miller & Herling (1956) found 16 α -hydroxytestosterone after perfusion of the parent compound through an isolated dog liver; Rao & Heard (1957) gave evidence for the 16 α -hydroxylation of progesterone after incubation with cell-free hog-adrenal preparations.

In view of the above, and of the possible role of neutral 16 α -hydroxy steroids as precursors of oestriol in its biosynthesis by human placenta (Ryan, 1958, 1959), an effort to obtain a better understanding of 16 α -hydroxylation by mammalian

tissues would seem justified. This paper describes the detection and isolation of 16 α -hydroxydehydroepiandrosterone after rat-liver slices were incubated with dehydroepiandrosterone. Reduced NADP increased the yield of this reaction. Liver slices from female rats, however, have little or no 16 α -hydroxylating ability.

MATERIALS AND METHODS

Abbreviation. DHA, Dehydroepiandrosterone.

Chemicals. DHA and deoxycorticosterone acetate were gifts from Laboratorios Drovysa, Barcelona, Spain, and Schering A.-G., Berlin, Germany. A sample of authentic 16 α -hydroxydehydroepiandrosterone was kindly given by Dr K. Fotherby. Blue tetrazolium was purchased from Dajac Laboratories, Philadelphia 24, Pa., U.S.A. Girard's reagent T was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A. Reduced NADP was prepared according to Kaplan, Colowick & Neufeld (1952) from the oxidized form (sodium salt) purchased from Sigma Chemical Co., St Louis 18, Mo., U.S.A.

All other chemicals were of A.R. quality. When solvents needed purification, standard procedures were followed (Vogel, 1951; Weissberger, Proskauer, Riddick & Toops, 1955).

Incubations. White rats of the Wistar strain bred in our animal colony were used. They were fed on Purina Laboratory Chow made by Casa Purina in Colombia on behalf of Ralston Purina Co., St Louis, Mo., U.S.A.

The rats were killed by ether and the livers quickly excised and immersed in ice-cold 0.9% NaCl solution. Slices of 0.5 mm. thickness were cut by means of a Stadie-Riggs microtome (A. H. Thomas Co., Philadelphia 5, Pa., U.S.A.) and weighed in a torsion balance. A typical incubation mixture consisted of 7.5 ml. of Krebs-Ringer phosphate solution, pH 7.4 (Umbreit, Burris & Stauffer, 1957), 2.5 mg. of DHA dissolved in 0.2 ml. of propylene glycol and 1 g. of slices. Similar mixtures, in which either DHA was added just after incubation or not at all, were used as blanks. The mixtures were then incubated at 38° in unstoppered flasks in a temperature-controlled water-bath shaker. After 0.5–4.5 hr., the contents of each flask were quantitatively transferred into a Potter-Elvehjem homogenizer, where a fine suspension was rapidly obtained. This was boiled for 1 min., and then centrifuged in a refrigerated centrifuge for 15 min. at 5° and 3000 rev./min. After removal of the supernatant, the residue was extracted three times with 20 ml. portions of hot aq. 85% (v/v) acetone. The combined acetone extracts were evaporated under reduced pressure to about 10 ml. This solution was pooled with the supernatant and extracted with 3 × 40 ml. of chloroform. After one washing with 0.2 vol. of saturated NaHCO₃ and two washings with 0.2 vol. of water, the chloroform extract was dried with anhydrous Na₂SO₄ and evaporated to dryness under reduced pressure in a water bath at 40°. From a methanolic solution of the dry residue appropriate portions were taken in duplicate for the analyses.

Urine extract. A total of 17.8 l. of urine from a healthy 31-year-old man was collected during 14 days. The urine was boiled, extracted and fractionated as described by Fotherby *et al.* (1957). The resulting ketonic fraction con-

tained 2.47 mg. of blue-tetrazolium-reducing material (relative to deoxycorticosterone acetate as standard) with the same mobility as 16 α -hydroxydehydroepiandrosterone in the paper chromatograms. This fraction was used as reference preparation during initial experiments before the pure steroid was available.

Miscellaneous. Melting points (Kofler stage) are uncorrected. For the colour reactions and for the spectra in conc. H₂SO₄ the methods described by Fotherby (1958) were used. A Beckman DU spectrophotometer was used with a diaphragm attachment to allow the use of 0.8 ml. Ultrasil cells (Pyrocell Manufacturing Co., New York, N.Y.). The paper-chromatographic methods of Bush (1952), modified by Fotherby *et al.* (1957), were followed, but room temperature was 26 ± 2°.

RESULTS

Experiments with liver slices of male rats. The ratio of substrate to tissue in these incubations was approximately 1:400. Portions of the extracts were analysed for blue-tetrazolium-reducing substances (Fotherby, 1958) as 16 α -hydroxydehydroepiandrosterone is one of the few known metabolic products of DHA that can react with blue tetrazolium. The results are given in Table 1.

The reducing material extracted from these incubations was compared with the reference preparation from human urine by chromatography on paper, side by side, in a modified solvent system B₅ (benzene-methanol-water; 10:7:3, by vol.) and in system B₂ (Bush, 1952; Fotherby *et al.* 1957). Pilot strips were cut out and sprayed with blue-tetrazolium reagent to locate reducing compounds. Apart from some reducing material running with the solvent front, only one spot was present in each preparation. Its R_F values in the two systems B₅ and B₂ were respectively 0.54 and 0.26. The corresponding zones in the remainder of the chromatogram were cut out and eluted with methanol. Portions were rechromatographed by exchanging the systems, i.e. in the modified B₅

Table 1. *Blue-tetrazolium-reducing substances after incubation of dehydroepiandrosterone with liver slices from male rats*

Rats weighed 212–362 g. Incubations lasted for 1 hr. at 38° in air as the gas phase. Results (relative to deoxycorticosterone acetate) are in μ g./g. of fresh tissue/hr.

Expt. no.	With DHA	Without DHA*	Net yield
1	38.3	9.2	29.1
2	81.5	18.4	63.1
3	72.0	18.3	53.7
4	51.1	14.1	37.0
5	75.2	47.4	27.8
6	85.8	44.7	41.1
Average of net yields			42.0

* DHA was added just after incubation.

system if the first chromatography took place in B_2 and vice versa. Again, only one reducing spot was seen with R_f values of 0.52 and 0.25. The blue-tetrazolium-reducing material from both preparations gave a positive Pettenkofer reaction (Fotherby, 1958). Both the spectra in sulphuric acid of the preparation from urine and of the incubation product exhibited a distinctive maximum at 378 $m\mu$, the general shape of the curves being almost identical but for the region around 300 $m\mu$, where the incubation product absorbed strongly.

The effects of modifying the length of the incubation period and the concentration of substrate, and the results of adding reduced NADP were studied (Table 2). The rate of production of reducing metabolites declined appreciably after incubation for 2 hr. The proportional increase in yield when a ratio of substrate to tissue greater than 1:400 was used was not high enough to justify exceeding this ratio in later experiments. It can also be seen that reduced NADP substantially increased the yield.

Sex difference and the effects of castration. Liver of female rats was not so effective as that of males in its ability to transform DHA into a blue-tetrazolium-reducing derivative (Table 3). The average net yield for female rats, 1.5 $\mu\text{g./g./hr.}$, is far lower than that for male rats (Table 1). Castration appeared to decrease liver activity somewhat, though it did not reach the low level of the females.

Isolation of 16 α -hydroxydehydroepiandrosterone. Livers from 50 male rats (weight 161–363 g.) were individually sliced and incubated as described above. The weights of the livers ranged from 5.2 to 13.0 g., and 20 mg. of DHA was used as substrate for all slices from each liver. After 2 hr. at 38°, the contents of each flask were stored at –40°. It took 12 days to complete all the incubations.

The combined incubation mixtures, 2.5 l., corresponding to 432 g. of liver and 1 g. of substrate, were homogenized in batches of about 300 ml. in a Waring Blendor. These suspensions were centrifuged at 1500 rev./min. and 5° for 30 min. The supernatants were decanted, pooled and extracted four times with 750 ml. of chloroform. The residues were repeatedly extracted with a total volume of 1.6 l. of hot aq. 85% (v/v) acetone which was evaporated under reduced pressure to a final volume of about 200 ml. This aqueous residue was extracted with chloroform (4 $\times$ 190 ml.), and the two extracts were combined and washed once with 0.2 vol. of saturated sodium hydrogen carbonate solution and three times with 0.2 vol. of water. The extract was then dried with 100 g. of anhydrous sodium sulphate, filtered and evaporated to dryness under reduced pressure. The residue, 2.574 g., contained 88 mg. of blue-tetrazolium-reducing material.

A discontinuous countercurrent distribution was next performed in 125 ml. separating funnels. The solvent system was methanol–water–benzene–chloroform (16:4:17:3, by vol.), and 40 ml. of both the upper (methanol) and lower (benzene) layers were used for the 23 transfers. The distribution was somewhat irregular, and the resolution im-

perfect, because emulsions occurred in some of the funnels. The contents of each funnel, after evaporation, were analysed for blue-tetrazolium-reducing material, 17-oxo steroids, and Pettenkofer-reacting substances (Fotherby, 1958). Pooled fractions 1–10 contained 557 mg. of total solids and 35 mg. of blue-tetrazolium-reducing material. Paper chromatography of this pooled material revealed

Table 2. *Results of variations in length of incubation (A), substrate concentration (B), and of addition of reduced nicotinamide-adenine dinucleotide phosphate*

The weights of male rats used ranged from 211 to 301 g. Substrate concentration is mg. of DHA/g. of fresh liver. Net yields of blue-tetrazolium-reducing products (relative to deoxycorticosterone acetate) are expressed as $\mu\text{g./g.}$ of fresh liver/hr. For other details, see Materials and Methods section.

Expt.	Length of incubation (hr.)	Concn. of substrate (mg.)	Net yield* ($\mu\text{g.}$)
A	0.5	2.5	60.0
A	1.0	2.5	57.8
A	1.5	2.5	52.7
A	2.0	2.5	50.5
A	3.0	2.5	45.3
A	4.5	2.5	33.6
B	2.0	0.4	5.5
B	2.0	0.8	15.2
B	2.0	1.2	20.3
B	2.0	2.5	26.5
B	2.0	5.0	37.5
C-1†	1.0	2.5	27.8
C-2‡	1.0	2.5	56.1
D-1†	1.0	2.5	41.1
D-2‡	1.0	2.5	58.6

* Averages of two separate incubations in experiments A and B.

† Without reduced NADP.

‡ With 2 μmoles of reduced NADP/g. of fresh tissue.

Table 3. *Experiments with rat-liver slices from female and castrated-male rats*

Figures represent blue-tetrazolium-reacting material in $\mu\text{g./g.}$ of fresh liver/hr. For conditions of incubation, see Material and Methods section. Interval between castration and experiment was 4 and 6 weeks respectively for rats 1 and 4; 5 weeks for rats 2 and 3.

Rat	With DHA	Without DHA*	Net yield
Female rats			
1	11.8	8.7	3.1
2	10.0	9.1	0.9
3	25.6	24.2	1.4
4	26.8	26.1	0.7
Castrated male rats			
1	73.1	28.3	44.8
2	37.8	11.6	26.2
3	61.3	41.0	20.3
4	29.5	15.6	13.9

* DHA was added just after incubation.

only one reducing spot with the mobility of 16 α -hydroxydehydroepiandrosterone.

After a separation with Girard's reagent T (Fotherby *et al.* 1957), 181 mg. of an oily ketonic fraction containing 18.2 mg. of blue-tetrazolium-reducing material was obtained. Chromatography of about 72% of this material on silica gel (Okada *et al.* 1959) yielded yellow crystals in part of the fraction eluted by 4% (v/v) ethanol in chloroform. After two crystallizations from methanol, the resulting white crystals, 8.9 mg., had m.p. 180–187° and did not depress the m.p., 168–175°, of the authentic sample of 16 α -hydroxydehydroepiandrosterone. Although Fotherby *et al.* (1957) first reported m.p. of 177–181° for this compound isolated from human urine, the synthetic compound obtained by Okada *et al.* (1959) had m.p. 182–186°.

Further evidence was obtained for the identity of the crystalline compound from its spectrum in concentrated sulphuric acid, colour reactions and paper chromatography. The infrared spectrum of the compound, measured in a potassium bromide pellet with a Perkin-Elmer model 21 instrument, was identical with that of an authentic specimen.

DISCUSSION

The isolation of 16 α -hydroxydehydroepiandrosterone reported in this paper is unequivocal evidence for the ability of rat liver to 16 α -hydroxylate *in vitro* a neutral steroid, thus corroborating other experiments with rabbit liver (Schneider & Mason, 1948) or dog liver (Axelrod *et al.* 1956). This evidence favours the view (Fotherby *et al.* 1957) that this compound might be an intermediate in the biosynthesis of androst-5-ene-3 β ,16 α ,17 β -triol, which was obtained by Schneider & Mason (1948) after incubating sodium dehydroepiandrosterone hemisuccinate with rabbit-liver slices.

The increased yield of 16 α -hydroxy compound with added reduced NADP is in keeping with the present conception (Samuels, 1960; Grant, 1960) that steroid hydroxylations are reactions catalysed by mixed-function oxidases requiring reduced NADP and molecular oxygen. It is not known whether the enzyme systems responsible for the 16 α -hydroxylation of neutral steroids, like DHA, and of phenolic steroids are identical. As far as the author is aware, no data have been published on the intracellular localization of the steroid 16 α -hydroxylase(s).

The sex difference in 16 α -hydroxylating ability reported in this paper is of interest. Axelrod *et al.* (1956) used a liver from a male dog in their perfusion experiments, in which 16 α -hydroxytestosterone was produced from testosterone. Hagopian & Levy (1958) and Breuer, Nocke & Pangels (1960) reported the isolation of oestriol after liver preparations from male rats were incubated with oestradiol-17 β . Sex differences in the way the

liver metabolizes some steroids are well known. For instance, Rubin (1957), working with liver homogenates, found different ratios of 3 β - to 3 α -hydroxylated products, resulting from the reduction of the 3-oxo group of androgens, depending upon the sex of the animal. In these experiments, there was also an additional species difference, since the ratio was higher for males than for females in the rat, rabbit, mouse and hamster, but lower in the guinea pig and turtle. Forchielli, Brown-Grant & Dorfman (1958) detected only the microsomal Δ^4 - α -hydrogenase in liver of female rats, whereas liver from male rats contained, in addition, a soluble Δ^4 - β -hydrogenase. Leybold & Staudinger (1959) reported a higher reducing activity with regard to 3-oxo-4,5-unsaturated steroids in liver microsomes isolated from female rats. They also reported that liver microsomes of male rats, but not of female animals, produced a more polar and unidentified steroid (probably by additional hydroxylation) from testosterone or 5 α -androstane-3 α ,17 β -diol in the presence of reduced NADP.

In the present studies, castration tended to decrease the 16 α -hydroxylating ability of male rat liver. Therefore, it would appear safe to assume that, whatever hormonal agent may be involved, its influence is not exerted on the actual hydroxylation, but rather on the amount or activity of the whole or part of the enzyme system involved.

SUMMARY

1. Incubation of dehydroepiandrosterone with liver slices from male rats results in the biosynthesis of a blue-tetrazolium-reducing metabolite which has been isolated and identified as 16 α -hydroxydehydroepiandrosterone.

2. Reduced nicotinamide-adenine dinucleotide phosphate has a stimulating effect on this reaction.

3. Little, if any, activity was shown by liver slices from female rats.

4. Castration appears to decrease 16 α -hydroxylating ability without its reaching, however, the low levels of the female.

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The Monosaccharide Units in Specific Glycolipids of *Mycobacterium avium*

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Lipids specific for species or type have been discovered in ethanol-ether extracts of strains of *Mycobacterium avium*, *Mycobacterium bovis* and the photochromogenic group (Runyon, 1955, 1959) of 'atypical' mycobacteria (Randall & Smith, 1953; Smith, Randall, Gastambide-Odier & Koevoet, 1957; Smith, Randall, MacLennan, Putney & Rao, 1960b). The specific substances were separated from non-specific lipids by column-adsorption chromatography and recognized by their distinctive infrared spectra. They were subsequently found to be glycolipids containing, characteristically, 6-deoxyhexoses and their methyl ethers (MacLennan, Smith & Randall, 1960, 1961; Smith *et al.* 1960b). The general name of 'mycoside' has been proposed (Smith, Randall, MacLennan & Lederer, 1960a) for this class of substances.

Mycoside A, characteristic of photochromogenic mycobacteria, contains 2-O-methylfucose, 2-O-methylrhamnose and 2,4-di-O-methylrhamnose; mycoside B, specific for *M. bovis*, contains 2-O-methylrhamnose alone (MacLennan *et al.* 1960, 1961). The lipid moieties of these substances con-

tain an aromatic alcohol in ester linkage with branched-chain fatty acids (Smith *et al.* 1957, 1960a, b).

Mycoside C, produced by *M. avium*, differs from other mycosides in that it contains a peptide (Smith *et al.* 1960a, b). It now appears that mycoside C is a mixture of peptido-glycolipids which can be resolved by adsorption chromatography into a number of components differing in their content of carbon, nitrogen and sugars, and in their optical activity (Jollès, Bigler, Gendre & Lederer, 1961).

The present study describes the isolation and identification of the sugars present in the glycolipid mixture, mycoside C, and in one of its components, mycoside C₁, recently isolated and characterized by Jollès *et al.* (1961).

The analysis of a lipopolysaccharide antigen of *Pseudomonas pseudomallei* is also described since it appeared (Chambon, Bourdet & Staub, 1957) that this substance might contain one or more of the uncommon sugars hitherto found only in bacteria of the order Actinomycetales.