Biochem. J. (1970) **119**, 803–804
Printed in Great Britain

Reduction of 5α-Androstane-3,16-dione by a Crystalline 20β-Hydroxy Steroid–Nicotinamide–Adenine Dinucleotide Oxidoreductase Preparation

By T. Pocklington,* J. Jeffery,† B. S. Middleditch‡ and C. J. W. Brooks
* *Department of Obstetrics and Gynaecology and* † *Department of Chemical Pathology, University of Aberdeen, Aberdeen AB9 2ZD, and* ‡ *Department of Chemistry, University of Glasgow, Glasgow, Glasgow W.2, U.K.*

(*Received* 4 *August* 1970)

Crystalline preparations of 20β-hydroxy steroid–NAD oxidoreductase (EC 1.1.1.53), an enzyme inducible in strains of *Streptomyces*, had 3α-hydroxy steroid–NAD oxidoreductase activity towards various 5α-steroids (Pocklington & Jeffery, 1968). 5α-Androstane-3,16-dione was a particularly good substrate, the maximum velocity and Michaelis constant for this compound being about 166 μmol/min per mg and 42 μM respectively, compared with about 5·0 μmol/min per mg and 300 μM respectively for 5α-androstane-3,17-dione under the same conditions. In the 3,17-dione, reduction was known to occur at the 3-position, and the question arose whether in the 3,16-dione reduction occurred at the 3-position or whether the preparation contained 16-hydroxy steroid–NAD oxidoreductase activity hitherto unrecognized. The amount of NADH consumed corresponded to reduction of only one oxo group (Pocklington & Jeffery, 1968); and kinetic measurements with mixtures of the 3,16-dione and 3,17-dione, though not diagnostic, permitted the view that these substrates possibly reacted at the same site (T. Pocklington & J. Jeffery, unpublished work). The characterization of the reduction product of the 3,16-dione is now reported.

5α-Androstane-3,16-dione (615 μg) was incubated at 25°C in phosphate buffer, pH 5.2 (20.07 ml), with enzyme (0.1 ml containing 0.02 mg of protein) and NADH (0.42 ml containing 2.1 mg), and the oxidation of the NADH followed spectrophotometrically at 340 nm, as previously described (Pocklington & Jeffery, 1968). When the reaction had gone to completion (3 min) the incubation mixture was extracted three times with diethyl ether (0.5 vol), the pooled extracts were washed three times with water (0.3 ml), and the ether was evaporated under reduced pressure. The residue was transferred by using ethanol and applied as a band to a t.l.c. plate (20 cm × 20 cm) spread with silica gel G (Pocklington & Jeffery, 1969). The plate was developed in hexane–ethyl acetate (1:1, v/v). R_F values were as follows: 5α-androstane-3,16-dione, 0.45; reduction product, 0.32; 3β-hydroxy-5α-androstan-16-one, 0.28. The reduction product was eluted from the gel with methanol (3 × 10 ml) and the pooled extracts were evaporated to dryness under reduced pressure. A specimen of this product was examined by g.l.c. [stationary phase 1% neopentylglycol succinate on Gas-Chrom Q (80–100 mesh); 240°C]. The retention time and retention factor of the reduction product (Table 1) were consistent with its formulation as 3α-hydroxy-5α-androstan-16-one.

The remainder of the reduction product was examined by combined g.l.c. [stationary phase 1% OV–17 on Gas Chrom Q (100–120 mesh); 200°C] and mass spectrometry (15 eV) with the LKB-Produkter model 9000 equipment. The fragmentation pattern of the trimethylsilyl ether of the reduction product was not identical with that of 3β-hydroxy-5α-androstan-16-one: both gave a molecular ion of *m/e* 362. Evidence for the absence of a 3-oxo function in the 'reduction product' was obtained from mass spectra of the bistrimethylsilyl ether. 3-Enol trimethylsilyl ethers of both 5α- and 5β-steroids give rise to characteristic prominent fragment ions of *m/e* 142 and 143 (Vetter, Walther, Vecchi & Cereghetti, 1969). Such ions were observed as intense peaks in the spectra (15 eV) of the enol trimethylsilyl ether ($M = 434$) derived from 17β-trimethylsilyloxy-5α-androstan-3-one and of the monoenol trimethylsilyl ether ($M = 360$) of 5α-androstane-3,16-dione (regarded as the 3-enol ether on chemical grounds and by virtue of its retention index). The trimethylsilyl ether/enol trimethylsilyl ether ($M = 434$)

Table 1. *G.l.c. retention characteristics of reduction product and related compounds*

Steroid	Retention time	Factor	
5α-Androstane-3,17-dione (I)	6 min 17 s	—	
3α-Hydroxy-5α-androstan-17-one (II)	4 min 35 s	(I)/(II)	1.37
3β-Hydroxy-5α-androstan-17-one (III)	5 min 25 s	(I)/(III)	1.16
5α-Androstane-3,16-dione (IV)	7 min 24 s	—	
Reduction product (V)	5 min 17 s	(IV)/(V)	1.40
3β-Hydroxy-5α-androstan-16-one (VI)	6 min 10 s	(IV)/(VI)	1.20

of the 'reduction product' showed no significant peaks at *m/e* 142 or 143. The mass spectrum was closely similar to that of the corresponding derivative of 3β-hydroxy-5α-androstan-16-one. It may be concluded that the 'reduction product' is 3α-hydroxy-5α-androstan-16-one, if the reasonable assumption is made that the skeletal structure remains unaltered in the reduction.

16α-Hydroxypregn-4-ene-3,20-dione, 3β,16α-dihydroxyandrost-5-en-17-one, 5α-androstane-3β,16α-diol and 3β-hydroxy-5α-androstan-16-one were not detected to undergo enzymic oxidation. A very slow oxidation of NADH was detected with 3β-hydroxy-5α-androstan-16-one under reducing conditions, and a similarly slow reduction of NAD^+ was detected with 5α-androstan-3β,16β-diol under oxidizing conditions. The possibility therefore exists that the enzyme preparation had a small amount of a limited type of 16β-hydroxy steroid–NAD oxidoreductase activity, though the rapid reduction of 5α-androstane-3,16-dione evidently occurred at the 3-position. 20β-Hydroxy steroid–NAD oxidoreductase-catalysed reduction of the 3-oxo group in 5α-androstane compounds containing an oxo group at either the 16- or the 17-position gives in each case the corresponding 3α-hydroxy compound.

We thank Professor I. MacGillivray and Professor S. C. Frazer for interest and encouragement, Professor W. Klyne and Dr D. N. Kirk for gifts of reference steroids from the Medical Research Council collection, and the Medical Research Council for a personal grant (to T.P.) and other support (to C.J.W.B.). The LKB9000 instrument was purchased under Science Research Council Grant no. B/SR/2398.

Pocklington, T. & Jeffery, J. (1968). *Eur. J. Biochem.* **7**, 63.

Pocklington, T. & Jeffery, J. (1969). *Eur. J. Biochem.* **9**, 142.

Vetter, W., Walther, W., Vecchi, M. & Cereghetti, M. (1969). *Helv. chim. Acta*, **52**, 1.