BBA 66571

STERIC, CHIRAL AND CONFORMATIONAL ASPECTS OF THE 3-HYDROXY- AND 20-HYDROXYSTEROID DEHYDROGENASE ACTIVITIES OF CORTISONE REDUCTASE PREPARATIONS

WILLIAM GIBB[a] AND JONATHAN JEFFERY[b]

[a]*Department of Obstetrics and Gynaecology and* [b]*Department of Chemical Pathology, University of Aberdeen, Aberdeen AB9 2ZD (Great Britain)*

(Received October 25th, 1971)

SUMMARY

The 3-hydroxysteroid dehydrogenase activity of cortisone reductase (20-dihydrocortisone:NAD^+ oxidoreductase, EC 1.1.1.53) was known to be 3α activity in the case of certain 5α-steroids in which 3α was 3R and axial. It is now shown to be 3α activity also in a 5α-steroid in which 3α is 3S and axial. In the 5β series, both 3α, 3R, equatorial activity and 3β, 3S axial activity are now described, though the compounds were very poor substrates. The view is discussed that interactions between the all-*trans*-5α-steroids and the enzyme distinguish the α side of the steroid from the β side; and that the angled A/B *cis*-steroids of the 5β series are either bound somewhat differently, or are possibly bound in more than one way.

The 20-hydroxysteroid dehydrogenase activity of cortisone reductase was known to be 20β activity in various cases where 20β was 20R. It is now shown to be 20β activity also in a case where 20β is 20S. In the case of 20 activity, the orientation around C-20 at the moment of hydrogen transfer is evidently fixed relative to both the steroid nucleus and the hydrogen donor or acceptor (presumably enzyme-bound NADH or NAD^+) even though rotation about the C-17/C-20 bond is possible.

INTRODUCTION

Cortisone reductase (20-dihydrocortisone:NAD^+ oxidoreductase, EC 1.1.1.53) transfers hydrogen from NADH to various 20-oxosteroids to give the 20β-hydroxy compounds[1,2] and to various 3-oxosteroids of the 5α series to give the 3α-hydroxy compounds (J. Jeffery, unpublished)[3]. The differences between these reactions are, perhaps, more striking than the similarities. Thus, the 3 and 20 positions are situated at opposite ends of the steroid molecule, and the reacting keto groups are endocyclic and exocyclic, respectively. The enzyme generally reduces 20-oxo groups much more efficiently than 3-oxo groups (though there are exceptions), and this is because the K_m values of the 20-oxosteroids are generally much lower (W. Gibb and J. Jeffery,

and I. H. White and J. Jeffery, unpublished). The V values are often higher for reduction of the 3-oxo compounds. Chemical reagents that reduce 20-oxo groups to 20β-hydroxy groups by a hydride transfer type of mechanism (*e.g.*, alkali metal borohydrides), reduce the less hindered 3-oxo groups of the 5α series more quickly, giving mainly the 3β-hydroxy compounds[4]. Complexes derived from chloroiridic acid and trimethyl phosphite, which give 3α-hydroxy compounds in the 5α series do not reduce the more hindered 20-oxo groups[5]. Nevertheless, the 20 and the 3 activities of cortisone reductase are not independent kinetically, and may depend upon the same active centre[6]. This enzyme therefore presents various points of interest in relation both to catalytic mechanisms and to steroid–protein interactions. In the work now described, three aspects of the specificity are considered, namely, the classical stereospecificity (α or β), the formal chiral specificity (R or S), and the conformational specificity (axial or equatorial) in the case of reaction at C-3.

MATERIALS AND METHODS

Steroids were obtained from the British Medical Research Council Steroid Reference Collection (Westfield College, London, Great Britain), Ikapharm (Ramat-Gan, Israel), Steraloids Limited (Croydon, Great Britain) and Syntex Corporation (Palo Alto, Calif., U.S.A.); pyridine nucleotides and crystalline enzyme preparations from Boehringer Mannheim G.m.b.H. (Mannheim, G.F.R.); solvents and general laboratory chemicals from Koch–Light Laboratories (Colnbrook, Buckinghamshire, Britain) and British Drug Houses Ltd. (Poole, Dorset, Great Britain); thin-layer adsorbants from E. Merck, A. G. (Darmstadt, G.F.R.) and 3,5-dihydroxypyrene-8,10-disulphonic acid disodium salt from Farbenfabriken Bayer A.G. (Leverkusen, G.F.R.).

Plates for thin-layer chromatography were spread with a slurry of silica gel $HF_{254+366}$ at a nominal thickness of 250 μm for analytical work, and 750 μm for preparative purposes; or with a slurry of aluminium oxide G (containing 20 mg of 3,5-dihydroxypyrene-8,10-disulphonic acid disodium salt per 100 g dry aluminium oxide) at 250 μm nominal thickness. The plates were allowed to dry overnight at room temperature (23°C) and were then activated for approximately 30 min at 80°C. The activity of the aluminium oxide plates declined with storage in an unsealed cabinet, and the plates were reactivated immediately before use if a test plate showed this to be necessary for the separation required. Partially deactivated plates were frequently satisfactory. The silica gel plates showed little change in activity over several days.

The steroids were located on both types of thin layer by viewing in shortwave ultraviolet (254 nm) or longwave ultraviolet (366 nm), when 4-ene-3-ones showed as dark regions, and the saturated steroids as light regions. These locations were confirmed by staining ketonic steroids with dinitrophenylhydrazine (saturated solution in ethanol or propan-2-ol freshly acidified with conc. HCl, 1 ml per 100 ml), and other steroids with phosphomolybdic acid (10% w/v in ethanol or 2-propanol).

The purity of steroid substrates was checked by melting point determination and thin-layer chromatography. In several cases small amounts of impurities were not readily removed by recrystallisation, and the compounds were purified by preparative thin-layer chromatography on silica gel. 5β-Androstane-3β,17β-diol (m.p.

163–165°C) was made by reducing a small sample of 3β-hydroxy-5β-androstan-17-one (M.R.C. steroid reference collection) with excess KBH_4 in ethanol at 23°C.

20β,21-Dihydroxypregn-4-en-3-one was prepared by reduction of 21-hydroxypregn-4-en-3,20-dione with $NaBH_4$ in cold methanol (*cf.* ref. 7). The 21-hydroxypregn-4-en-3,20-dione (526 mg) was dissolved in methanol (90 ml) at −20°C. $NaBH_4$ (103 mg) was dissolved in methanol (25 ml) at −20°C. The steroid solution was then heated to 0°C by means of an ice–water bath, and the $NaBH_4$ solution added in 5-ml portions at intervals of 10 min. Further $NaBH_4$ (100 mg) freshly dissolved in methanol (25 ml) at −20°C, was added in two portions, 10 ml after 150 min, and 15 ml after 160 min. The course of reaction was followed by thin-layer chromatography of samples on silica gel. The plates were examined in ultraviolet light (254 nm) for 4-en-3-one compounds, and were exposed to HCl vapour to colour any 3-hydroxy-4-enes, which (like the 3-hydroxy-5-enes) give a pink colour[8]. Thus, after 170 min, the reaction mixture contained mainly 20β,21-dihydroxypregn-4-en-3-one, a little residual starting material and a small amount of a 3-hydroxy-4-ene. After 240 min, glacial acetic acid was added dropwise until the pH was about 6, and the solvents removed by rotary evaporation, leaving a gum. Crystals formed overnight, and four recrystallisations from acetone gave material m.p. 157–165°C (200 mg), and, from the mother liquors, material m.p. 127–153°C (153 mg). Both materials were mainly 20β,21-dihydroxypregn-4-en-3-one contaminated with starting material and 3-hydroxy-4-ene. Recrystallisations from acetone, or preparative thin-layer chromatography and crystallisation from acetone gave material m.p. 165–168°C, homogeneous by thin-layer chromatography.

The course of the enzymatic reaction was followed spectrophotometrically at 340 nm as described elsewhere[6]. For preparation of the reaction products, the reaction was allowed to proceed at 25°C until the amount of co-enzyme transformed corresponded to at least 80% of the steroid substrate, and the reaction rate had become inappreciable. The time required ranged from less than 1 h to several hours (*e.g.* overnight) and the conditions used are summarised in Table I. After reaction, the

TABLE I

INCUBATION CONDITIONS AND EXTENT OF REACTION FOR PREPARATIVE EXPERIMENTS

Substrate concentration	*Co-factor Concentration*	*Enzyme (μg/ml)*	*pH*	*Dimethyl sulphoxide (%, v/v)*	*Total vol. (ml)*	*% reaction*
17β-Hydroxy-2α-methyl-5α-androstan-3-one 50 μM	NADH 150 μM	8	5.4	10	250	90
5β-Androstan-3,17-dione 25 μM	NADH 150 μM	92	5.4	10	10.8	80
17β-Hydroxy-5β-androstan-3-one 25 μM	NADH 150 μM	9.2	5.4	10	10.8	90
5β-Androstane-3α,17β-diol 25 μM	NAD^+ 1 mM	9.2	8.0	10	10.8	85
5β-Androstane-3β,17β-diol 25 μM	NAD^+ 1 mM	23	8.0	10	10.8	85
21-Hydroxypregn-4-ene-3,20-dione 50 μM	NADH 150 μM	3	5.4	20	8.1	90

solutions were extracted with diethyl ether, the ether extracts washed with water, dried with Na_2SO_4 and the ether removed by rotary evaporation. The residue was dissolved in a small volume of acetone or diethyl ether, and portions examined by thin-layer chromatography.

RESULTS

The following 3α-hydroxy-5α-androstane compounds were substrates (0.1 M sodium phosphate buffer, pH 7.0; 1 mM NAD^+; 37 μg enzyme/ml; 25 μM steroid; 10% v/v dimethyl sulphoxide): 5α-androstane-3α,17β-diol and 3α-hydroxy-5α-androstan-17-one. Under the same conditions, no reaction was detected with 5α-androstane-3β,17β-diol and 3β-hydroxy-5α-androstan-17-one, nor with 2α-methyl-5α-androstane-3β,17β-diol even in the presence of 93 μg enzyme/ml. In the presence of NADH (Table I), 17β-hydroxy-2α-methyl-5α-androstan-3-one was a substrate, and the product was not 2α-methyl-5α-androstane-3β,17β-diol (Table II) but was a slightly

TABLE II

CHARACTERISATION OF THE REACTION PRODUCTS BY THIN-LAYER CHROMATOGRAPHY

Material and system	*No. of spots*	R_F
Silica gel, benzene–acetone (2:1, v/v)		
Substrate: 17β-hydroxy-2α-methyl-5α-androstan-3-one		0.71
Product		0.62
Product *plus* 2α-methyl-5α-androstane-3β,17β-diol	two spots	0.62 and 0.57
On a separate plate:		
5α-Androstane-3α,17β-diol		0.58
5α-Androstane-3β,17β-diol		0.51
Product appears to be 2α-methyl-5α-androstane-3α,17β-diol		
Silica gel, hexane–ethyl acetate (1:1, v/v)		
Substrate: 5β-androstane-3,17-dione		0.65
Product*	two spots	0.52 and 0.39
On a separate plate:		
3β-Hydroxy-5β-androstan-17-one		0.62
3α-Hydroxy-5β-androstan-17-one		0.47
Product *plus* 3α-hydroxy-5β-androstan-17-one	two spots	0.49 and 0.62
On a separate plate:		
3β-Hydroxy-5β-androstan-17-one		0.51
3α-Hydroxy-5β-androstan-17-one		0.36
Product *plus* 3β-hydroxy-5β-androstan-17-one	two spots	0.38 and 0.52
On a separate plate:		
Product *plus* 3β-hydroxy-5β-androstan-17-one	two spots	0.60 and 0.44
Product *plus* 3α-hydroxy-5β-androstan-17-one	two spots	0.61 and 0.46
Product *plus* 17β-hydroxy-5β-androstan-3-one	three spots	0.61, 0.54 and 0.46
Product *plus* 17α-hydroxy-5β-androstan-3-one	three spots	0.61, 0.54 and 0.46
Product appears to be both 3α- and 3β-hydroxy-5β-androstan-17-one		
Silica gel, hexane–ethyl acetate (2:3, v/v)		
Substrate: 17β-Hydroxy-5β-androstan-3-one		—
5β-Androstane-3β,17β-diol		0.59
5β-Androstane-3α,17β-diol		0.42
Product *plus* 5β-androstane-3β,17β-diol	two spots	0.43 and 0.59
On a separate plate:		
5β-Androstane-3β,17β-diol		0.57

(Continued on p. 17)

TABLE II *(Continued)*

Material and system	*No. of spots*	R_F
5β-Androstane-3α,17β-diol		0.39
Product *plus* 5β-androstane-3α,17β-diol	one spot	0.41
Product appears to be 5β-androstane-3α,17β-diol		
Silica gel, hexane–ethyl acetate (2:3, v/v)		
Substrate: 5β-Androstane-3α,17β-diol		—
5β-Androstane-3,17-dione		0.64
17β-Hydroxy-5β-androstan-3-one		0.42
Product *plus* 3α-hydroxy-5β-androstan-17-one	two spots	0.40 and 0.45
On a separate plate:		
5β-Androstane-3,17-dione		0.64
3α-Hydroxy-5β-androstan-17-one		0.41
Product *plus* 17β-hydroxy-5β-androstan-3-one	one spot	0.44
Aluminium oxide, hexane–ethyl acetate (2:1, v/v)		
Product *plus* 17β-hydroxy-5β-androstan-3-one	one spot	0.43
Product *plus* 3α-hydroxy-5β-androstan-17-one	two spots	0.44 and 0.31
3α-Hydroxy-5β-androstan-17-one *plus* 5β-androstane-3,17-dione	two spots	0.15 and 0.80
Product appears to be 17β-hydroxy-5β-androstan-3-one		
Silica gel, hexane–ethyl acetate (1:1, v/v)		
Substrate: 5β-Androstane-3β,17β-diol		—
Product*	one spot	0.40
On a separate plate:		
Product *plus* 17β-hydroxy-5β-androstan-3-one	one spot	0.47
3β-Hydroxy-5β-androstan-17-one		0.57
5β-Androstane-3,17-dione		0.69
5β-Androstane-3β,17β-diol		0.45
On a separate plate:		
Product *plus* 3β-hydroxy-5β-androstan-17-one	two spots	0.49 and 0.58
17β-Hydroxy-5β-androstan-3-one		0.48
5β-Androstane-3,17-dione		0.71
5β-Androstane-3β,17β-diol		0.48
Product appears to be 17β-hydroxy-5β-androstan-3-one		
Silica gel, hexane–ethyl acetate (10:43, v/v)		
Substrate: 21-Hydroxypregn-4-ene-3,20-dione		—
20β,21-Dihydroxypregn-4-en-3-one		0.44
Product *plus* 20β,21-dihydroxypregn-4-en-3-one	one spot	0.44
Product *plus* 20α,21-dihydroxypregn-4-en-3-one	two spots	0.43 and 0.37
Aluminium oxide, ethyl acetate–acetone (80:32, v/v)		
Product	one spot	0.32
Product *plus* 20β,21-dihydroxypregn-4-en-3-one	one spot	0.32
Product *plus* 20α,21-dihydroxypregn-4-en-3-one	two spots	0.32 and 0.16
Product appears to be 20β,21-dihydroxypregn-4-en-3-one		

* Plate stained with 2,4-dinitrophenylhydrazine showing presence of a keto group in the product.

less polar compound, presumably 2α-methyl-5α-androstane-3α,17β-diol. Thus, for these 5α-androstane compounds the principal and possibly the only activity was 3α-hydroxysteroid:NAD^+ oxidoreductase activity.

5β-Androstane-3α,17β-diol and 5β-androstane-3β,17β-diol were both substrates (Table I), though very poor substrates, the initial velocities being roughly 0.029 and 0.0023 μmole/min per mg enzyme, respectively. The products in both cases appeared identical with 17β-hydroxy-5β-androstan-3-one (Table II), and were not 3α-hydroxy-5β-androstan-17-one or 3β-hydroxy-5β-androstan-17-one.

17β-Hydroxy-5β-androstan-3-one was a substrate in the presence of NADH (Table I). The reaction was slow, the initial rate being roughly 0.042 μmole/min per mg enzyme. The only reaction product detected appeared to be 5β-androstane-3α,17β-diol (Table II): none of the 3β isomer was detected, but the possible presence of a small amount of it was not excluded.

5β-Androstane-3,17-dione was a very poor substrate (Table I), the initial rate of reduction being about 0.0044 μmole/min per mg enzyme. Two products of reaction were detected (Table II), apparently 3α-hydroxy-5β-androstan-17-one and 3β-hydroxy-5β-androstan-17-one. The reaction products were not 17α-hydroxy-5β-androstan-3-one or 17β-hydroxy-5β-androstan-3-one (Table II). The product thought to be 3α-hydroxy-5β-androstan-17-one was present in somewhat larger amount than the product thought to be 3β-hydroxy-5β-androstan-17-one, as judged visually by the intensity of the colour with phosphomolybdic acid. The amounts of the two products were, however, not greatly dissimilar. It therefore appeared that for these 5β-androstane compounds there was both 3α- and 3β-hydroxysteroid:NAD^+ oxidoreductase activity.

20β,21-Dihydroxypregn-4-en-3-one was a substrate (0.1 M sodium phosphate buffer, pH 8.0; 2 mM NAD^+; 45 μM steroid; 20% (v/v) dimethyl sulphoxide; 3.7 μg enzyme/ml), reaction corresponding to about 72% conversion in about 3 min. Under the same conditions, but with 18.5 μg enzyme/ml, no reaction was detected with 20α,21-dihydroxypregn-4-en-3-one. Only one product was detected from the enzyme reduction of 21-hydroxypregn-4-ene-3,20-dione (Table I). This could not be distinguished from 20β,21-dihydroxypregn-4-en-3-one under conditions which would have clearly distinguished the 20α isomer. In this case, therefore, the principal and possibly the only activity was 20β-hydroxysteroid:NAD^+ oxidoreductase activity.

DISCUSSION

In the 5α-androstane compounds studied, the hydroxyl groups oxidised had the 3α configuration, and the conformation axial. The formal chirality was 3*S* in the case of the 2α-methyl compound (because the 2-methyl substituent gives C-2 priority over the unsubstituted C-4), and 3*R* in the other cases. Towards the 5β-androstane compounds, there was both 3α- and 3β-hydroxysteroid dehydrogenase activity, with some predominance of the former. In these 5β compounds, the 3α-hydroxyl groups were 3*R* and equatorial, and the 3β-hydroxyl groups were 3*S* and axial. The 5β compounds were very poor substrates, and the reaction rates were so slow, and the apparent K_m values so high compared with the solubilities of the substrates, that it has not so far been possible to show by kinetic studies whether or not the 5α and 5β compounds react at different centres. In the absence of such evidence it seems reasonable to discuss the activities in terms of one active centre, particularly since the 3α and 20β activities are closely related[6]. Thus, the characteristics of the 3 activity can be summarised as follows. The enzyme does not add or remove only equatorial hydrogen; it does not add or remove only *R* hydrogen; and it does not add or remove only β hydrogen, though this last mentioned possibility may usually be the favoured course. One interpretation would be that the enzyme distinguishes the α from the β face of the steroid, and that, while this distinction is well made among the all-*trans* 5α compounds, the angled *A/B cis* compounds of the 5β series are bound somewhat

differently and can possibly be bound in more than one way. This would require that the binding region is bigger than a steroid molecule, and/or flexible; and that the binding forces are not highly specific. There is, indeed, other evidence broadly consistent with such a view (W. Gibb and J. Jeffery, unpublished), and the concept of a hydrophobic binding region in which relatively non-specific interactions occur over much of the steroid molecule makes an attractive hypothesis. Nevertheless, 5α-androstane-3,16-dione was a particularly good substrate[6], evidently because of some influence of the 16-oxo group; and in the present study, a 17β-hydroxy or 17-oxo group in the 5β compounds influenced the relative amounts of 3α- and 3β-hydroxy compounds formed. A simple polar group remote from the reacting 3-oxo function can therefore greatly influence the enzymic reaction.

Reactions catalysed by this enzyme at C-20 were shown to be specific for 20β-hydroxy compounds in the case of two 17α,21-dihydroxysteroids[1] and various 17,21-deoxysteroids[2]. In all these cases, the 20β compounds were 20R. In the 17-deoxy-21-hydroxy series, the 20β-hydroxy compounds are 20S because the 21-hydroxyl group gives C-21 priority over C-17. 11β,21-Dihydroxypregn-4-ene-3,20-dione was a known substrate[9], but characterisation of the product appeared not to have been described. The steric course of reaction was investigated in the present study, using 21-hydroxypregn-4-ene-3,20-dione and the corresponding 20-hydroxy compounds. Only 20β activity was detected. Thus, among these compounds, it is the 20β configuration and not the 20R chirality which the enzyme distinguishes. This seems to imply that at the moment of hydrogen transfer, the 20-oxo group has a fixed orientation relative to both the steroid nucleus and the hydrogen donor or acceptor (presumably enzyme-bound NADH or NAD^+). This is not necessarily the conformation favoured in the free steroid (in which the 20-oxo group is thought to project toward the β face of the molecule and the C-20/C-21 bond to eclipse the C-17/17α-H bond[10,11]) but this would in fact allow hydrogen transfer to the 20α position by the less-hindered approach (as occurs, for example, during reduction with $NaBH_4$).

A small point concerning nomenclature merits comment. The I.U.P.A.C. rules[12] describe the R and S system and in Note 1, to Rule 2S-1.6 state that "the 20α/20β nomenclature is continued because of long tradition". So far as is known at present, the 20 activity of this enzyme can be described as 20β-hydroxysteroid activity, but could not be described either as 20R- or as 20S-hydroxysteroid activity. This provides a reason additional to "long tradition" for continuing the 20α/20β nomenclature.

ACKNOWLEDGEMENTS

We thank Prof. S. C. Frazer and Prof. I. MacGillivray for their interest and encouragement; Dr H. J. Ringold for kindly donating the 2α-methyl compounds; Messrs Boehringer Mannheim for gifts of enzyme and co-factor; Prof. W. Klyne and Dr D. N. Kirk for kindly providing steroids from the British Medical Research Council Steroid Reference Collection; and the British Medical Research Council for an award for training in research methods (to W.G.).

REFERENCES

1 H. J. Hübener and C. O. Lehmann, *Z. Physiol. Chem.*, 313 (1958) 124.
2 H. D. Henning and J. Zander, *Z. Physiol. Chem.*, 330 (1962) 31.

3 T. Pocklington, J. Jeffery, B. S. Middleditch and C. J. W. Brooks, *Biochem. J.*, 119 (1970) 803.
4 W. G. Dauben, G. J. Fonken and D. S. Noyce, *J. Am. Chem. Soc.*, 78 (1956) 2579.
5 P. A. Browne and D. N. Kirk, *J. Chem. Soc. (C)*, (1969) 1653.
6 W. Gibb and J. Jeffery, *Eur. J. Biochem.*, 23 (1971) 336.
7 J. K. Norymberski and G. F. Woods, *J. Chem. Soc.*, (1955) 3426.
8 J. Jeffery, *J. Chromatogr.*, 59 (1971) 216.
9 H. J. Hübener, in H. U. Bergmeyer, *Methods of Enzymatic Analysis*, Academic Press, New York, 1963, p. 484.
10 L. B. Keir, *J. Med. Chem.*, 11 (1968) 915.
11 W. G. Cole and D. H. Williams, *J. Chem. Soc.*, (1968) 1849.
12 I.U.P.A.C. Commission, *Eur. J. Biochem.*, 10 (1969) 1.