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REDUCTION OF STEROIDAL 20-OXO-21-OIC ACIDS TO STEROIDAL 20β-HYDROXY-21-OIC ACIDS BY 20β-HYDROXYSTEROID DEHYDROGENASE

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SUMMARY

A simple stereospecific synthesis of steroidal 20β-hydroxy-21-oic acids is described. The 20β hydroxysteroid dehydrogenase of Streptomyces hydrogenans reduced 3,20-dioxo-4-pregnen-21-oic acid to 20β-hydroxy-3-oxo-4-pregnen-21-oic acid. Other steroidal 11-deoxy or 11,20-dioxy acids were substrates of the enzyme; those containing the 11β-hydroxy group were not.

INTRODUCTION

Specific enzymes have been isolated from human [1] and hamster [2] liver which catalyze the conversion of the steroidal ketol side chain to the 20-hydroxy-21-oic acid side-chain. These steroidal 20-hydroxy-21-oic acids can exist in two enantiomeric configurations, determined by the asymmetry at position 20. Both 20α and 20β isomers of 3α,17,20-trihydroxy-11-oxo-pregnen-21-oic acid and 3α,11β,17,20-tetrahydroxy-pregnen-21-oic acid have in fact been isolated from human urine [1]. In order to rigorously characterize these metabolic products, unequivocal stereospecific synthesis of the two possible epimers are needed. In this paper I described a simple, specific method for the preparation of steroidal 20β-hydroxy-21-oic acids.

MATERIALS AND METHODS

The 20β-hydroxysteroid dehydrogenase of Streptomyces hydrogenans was purchased as a crystalline suspension in 3.2 M ammonium sulfate from Boehringer-Mannheim Corp. NADH and NAD^+ were bought from P-B Biochemicals, Inc., and corticosteroids from Steraloids, Inc. All steroids were found to be chromatographically homogeneous on thin layer plates in appropriate solvent systems. The steroids were oxidized to 21-dehydrosteroids as described by Monder and Furfine [3] and to 20-oxo-21-oic acids by a method described earlier [4] using chromium trioxide for 17-deoxy steroids and methylene blue for 17-hydroxy steroids.

Reduction of all steroids by NADH was monitored continuously at 340 nm on a Gilford Model 2000 spectrophotometer. Spectra in the visible and U.V. were scanned on a Cary Model 15 instrument. Infrared spectra were obtained in KCl pellets with a Perkin-Elmer Model 221 instrument. Buffers used were either 0.1 M sodium phosphate or 0.1 M 2-(N-morpholino)-ethanesulfonic acid (MES).

RESULTS AND DISCUSSION

Reduction of 3,20-dioxo-4-pregnen-21-oic acid (pregnenoic acid) by 20β-hydroxysteroid dehydrogenase proceeded readily under the conditions described by Hubener [5]. The rate of reaction, measured as NADH oxidation at 340 nm was optimal at pH 5.1 in MES buffer (Fig. 1). At this pH value, reduction proceeded in a linear manner with 1.5×10^{-4} M pregnenoic acid until more than 50% of the substrate was reduced. The initial rate of reaction was directly

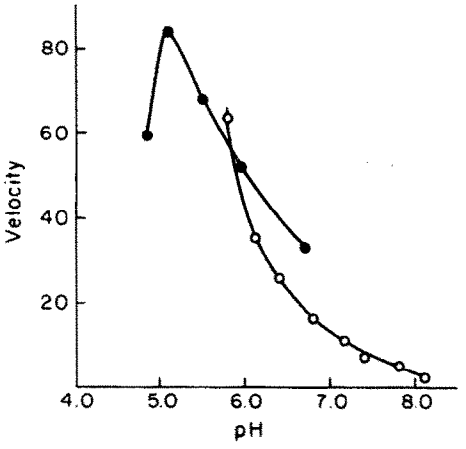

Fig. 1. Effect of pH on velocity. The incubation mixture contained 2.85 ml of 0.01 M buffer; 50 μg of enzyme; 0.33 μmol of NADH; 0.58 μmol of 3,20-dioxo-4-pregnen-21-oic acid in a final volume of 3.0 ml. ●——● 2-(N-morpholino)-ethanesulfonic acid; O——O sodium phosphate. Ordinate refers to initial velocity.

Table 1. Kinetic constants of various substrates and cofactors*

Substrate	Apparent K_M	V_{max}
	M	μmol/h/μg enzyme
(1) 11β,17-dihydroxy-3,20-dioxo-4-pregnen-21-oate	—	—
(2) 11-hydroxy-3,20-dioxo-4-pregnen-21-oate	—	—
(3) 17-hydroxy-3,20-dioxo-4-pregnen-21-oate	$6.4 \pm 2.2 \times 10^{-4}$	0.48 ± 0.12
(4) 17-hydroxy-3,11,20-dioxo-4-pregnen-21-oate	$4.9 \pm 0.6 \times 10^{-4}$	0.28 ± 0.03
(5) 3,20-dioxo-4-pregnen-21-oate	$1.4 \pm 0.6 \times 10^{-4}$	0.34 ± 0.10
(6) Progesterone	$6.1 \pm 0.4 \times 10^{-6}$	0.11 ± 0.01
(7) NADH	$3.9 \pm 1.4 \times 10^{-4}$	—
(8) NADPH	—	—

*The incubation mixtures contained 93 μmol of 0.1 M 2-(N-morpholino)-ethanesulfonic acid, pH 5.1, 100 μg (0.13 μmol) of NADH, 1 μg of enzyme protein, and steroid in 30 μl of methanol in a final volume of 1.0 ml. Temperature was 28°C. Maximum velocities and Michaelis constants were obtained from double reciprocal plots by varying the substrates (expressed as mmolar concentrations) as follows: substrate 1 (0.160 to 0.401); substrate 2 (0.166 to 0.416); substrate 3 (0.166 to 0.416); substrate 4 (0.160 to 0.400); substrate 5 (0.044 to 0.218); progesterone (0.0095 to 0.191); NADH with substrate 5 at 0.218 mmolar as cosubstrate (0.020 to 0.197). All values were obtained from initial velocities from which blank values with no steroid glyoxylate were subtracted. Dashes indicate no measurable reaction.

proportional to enzyme concentration within the range of 3 to 15 μg protein per ml of incubation mixture.

Not all 20-oxo-21-oic acids were reduced by 20β-hydroxysteroid dehydrogenase. Only those which lacked the 11β-hydroxy group were substrates. Table 1 shows that the active steroidal keto acids had to be present at 100- to 1000-fold greater concentrations than progesterone in order to achieve half maximal velocities, but the maximum rates of reduction were in each case two to five times greater than that of progesterone.

The K_M value for progesterone at pH 5.1 is close to that recorded in the literature for this substrate at pH 6.5 [6]. The K_M value for NADH is 100–500 times less at pH 5.1 than at pH 6.5 [6]. The inefficient binding of NADH may account for the relatively slow rate of reduction of progesterone, a normally highly efficient substrate, at pH 5.1. None of the substrates were reduced with NADPH.

In a system containing 0.167 mM NAD and 0.29 μmol of pregnenoate at pH 5.1, oxidation of NADH measured as decrease in absorbancy at 340 nm proceeded to 65% of theory in 12 h. Further decrease did not occur when more enzyme and NADH was added. There seemed to be occasionally a small increase in absorbancy under these conditions. The results suggested that equilibrium may have been achieved between the 20-oxo and 20-hydroxy forms. Therefore, an attempt was made to see if the reaction is reversible.

20β-hydroxy-3-oxo-4-pregnen-21-oic acid was prepared as described below, and was incubated with NAD^+ and enzyme in phosphate buffer in the range of pH 5.75 to 8.1, or MES buffer, pH range 3.7 to 6.5. As the pH dropped from 6.5 to 3.7, there was a progressively faster rate of increase in absorbancy at 340 nm. Surprisingly, no spectral maximum was seen at 340 nm indicating lack of NAD^+ reduction. Absorbancy of the product showed a continuum of increasing intensity from 500 to 320 nm with no spectral maximum anywhere. A mixture of the hydroxy acid and enzyme increased in absorbancy at 340 nm at a rate equal to that of the complete mixture, and yielded an indentical spectral profile. Visual inspection revealed a faint opalescence in the cuvette. Interaction of 20β-hydroxy steroid acid with enzyme resulted in the formation of an insoluble complex which was enzymically inactive. Therefore, apparent incomplete reduction of keto acids as measured at 340 nm was probably caused in part by inactivation of the enzyme by product and by the optical interference of the product-enzyme complex. These effects were observed at pH 5.1 as well as pH 3.7. From these results it was concluded that the net decrease in absorbancy at 340 nm is not a true reflection of the total reduction of steroid. It was therefore decided that absorbancy measurements at 340 nm could not be used to monitor the extent of reduction of steroid with NADH quantitatively, although it was an accurate indicator of the initial rate of the reaction.

Large scale reduction of DOC glyoxylate was performed as follows: Into each of five 50 ml flasks was placed 30 ml of 0.1 M MES, pH 5.1, 5 mg of NADH in 1 ml of water, 5 mg of 3,20-dioxo-4-pregnen-21-oic acid in 1 ml of methanol and 300 μg of 20β-hydroxysteroid dehydrogenase. Progress of the reaction was followed at 340 nm. A second charge of 300 μg of enzyme was added after 2 h. After 20 h at approx. 27°C (room temperature), the incubation mixtures were combined, adjusted to pH 2 with 3 N HCl, and extracted exhaustively with ethyl acetate. Water was removed from the organic phase with sodium sulfate, and the solvent was removed *in vacuo*. The residue was taken up in methanol and chromatographed on 2 mm thick silica gel plates with chloroform–methanol–formic acid (84:16:1, by vol.) as the developing solvent. The component with mobility corresponding to synthetic 20-hydroxy-3-oxo-4-pregnen-21-oic acid was transferred to a Soxhlet apparatus and extracted

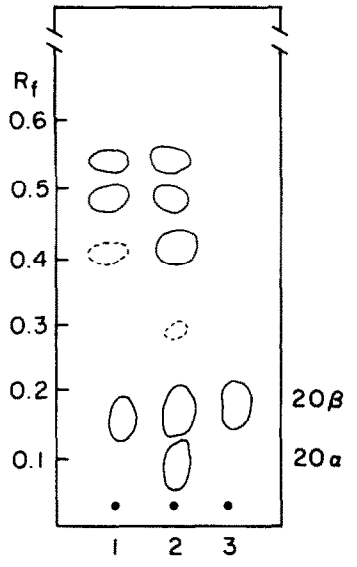

Fig. 2. Chromatographic analysis of stereochemical configuration at position 20 of 20β-hydroxy-3-oxo-4-pregnen-21-oic acid. The enzymatically prepared product was converted to 20β,21-dihydroxy-40-pregnen-3-one by reducing the acid with lithium aluminum hydride in tetrahydrofuran under reflux followed by reoxidation of the resulting 3-ol-5-ene with manganese dioxide. Borate impregnated thin layer plates were prepared by dipping 0.3 mm thick silica gel plates in 0.1 M sodium borate, pH 9.2, then drying the plates for 1 hr at 90°. Plates were developed at room temperature (26°C) with ethyl acetate. Channel 1, reduced product from enzymically synthesized 20-hydroxy acid; channel 2, reduced product from chemically synthesized 20 hydroxy acid; channel 3, 20β,21-dihydroxy-4-pregnen-3-one standard.

over a period of several hours with acetone. The acetone was removed with a stream of warm nitrogen to give a homogeneous crystalline product weighing 19.3 mg.

Anal. Calc. for $C_{21}H_{30}O_4.H_2O$; C, 69.2; H, 8.8 Found: C, 69.7; H, 8.4. m.p. 191–192°C (sinters at 187°C); I.R. (KBr) 2.95, 3.4, 4 (shoulder), 5.8, 6.1, 7.2, 9.2 μ. Spectrum corresponded to chemically synthesized acid.

The acid was treated overnight with diazomethane in ether. Conversion to 20-hydroxy-3-oxo-4-pregnen-21-oic acid 21 methyl ester was quantitative.

Anal. Calc. for $C_{22}H_{32}O_4.0.5\ H_2O$: C, 71.5; H, 9.0 Found: C, 71.5; H, 8.9 ir (KBr) 2.95, 3.4, 6.0, 6.3, 7.1, 9.5, 9.8 μ. Spectrum corresponded to synthetic ester.

Configuration of the steroid at carbon 20 was established by converting the acid to 20,21-dihydroxy-4-pregnen-3-one and comparing the chromatographic mobility of the product with that of the authentic epimeric diols. Two milligrams of 20β-hydroxy-3-oxo-4-pregnen-21-oic acid was refluxed with 14 mg lithium aluminum hydride in dry tetrahydrofuran for 75 min. Excess reducing agent was destroyed with 1 ml ethyl acetate. Solids were coagulated with a few drops of saturated aqueous sodium sulfate, followed by 50 mg of anhydrous magnesium sulfate. The reduced steroid in dry chloroform was stirred overnight with manganese dioxide, filtered, concentrated, and chromatographed on thin layer plates with authentic 20β,21-dihydroxy-4-pregnen-3-one and with the 20α,21-diol obtained from reduction of the 20-hydroxy acid resulting from cupric acetate catalyzed rearrangement of 3,20-dioxo-4-pregnen-21-al [7]. The results, reproduced in Fig. 2, conclusively show that the product derived from the reduction of 3,20-dioxo-4-pregnen-21-oic acid is 20β-hydroxy-3-oxo-4-pregnen-21-oic acid.

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