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Steroid-Protein Interaction: Substrate Specificity of 20β-Hydroxysteroid Dehydrogenase[1]

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The reactivities of 20β-hydroxysteroid dehydrogenase [EC 1.1.1.53] from *Streptomyces hydrogenans* towards 64 kinds of steroids having the pregnan-20-one skeleton were investigated. The presence and nature of a substituent around the reacting 20-oxo group of the steroid played a decisive role in the interaction with the catalytic site of the enzyme. In general, steroids having bulky group(s) at C-21 and/or C-17 did not act as substrates, though steroids having a bulky group at C-3 or C-11 were utilized by the enzyme. The existence of a substituent at C-16 caused 20-oxo steroids to lose their reactivity almost completely, but the existence of a substituent at C-3, C-6, C-9, C-11, C-17 or C-21 did not. The configurational relationship between the plane of the steroid ring and the C-17 side chain might also be important in steroid recognition by the enzyme. Among the non-substrate steroids, 16β-methylpregn-4-ene-3,20-dione was a competitive inhibitor with respect to both steroid substrate and coenzyme. It was inferred that the steroid ring of the inhibitor molecule competed with the substrate binding process, while the 16β-methyl substituent competed with the coenzyme action. When 16α-methylpregn-4-ene-3,20-dione, pregn-4-ene-3,20-dione and 17,21-dihydroxypregn-4-ene-3,11,20-trione were used as substrates, the apparent K_m values for NADH were 13.97, 3.91 and 4.07 μM, respectively. The presence of a substituent at C-16 unfavorably affected the apparent K_m for NADH, but substituents at C-21, C-17 or C-11 did not. These results suggest that the coenzyme may be located in the vicinity of C-16 of the steroid molecule in the ternary complex, steroid-coenzyme-enzyme.

Keywords——20β-hydroxysteroid dehydrogenase; substrate specificity; steroids; steroid-protein interaction; 16β-methylprogesterone; K_m for NADH; pregnan-20-one derivative; high-performance liquid chromatography

Various steroids (intrinsic hormones or drugs) are transported to target organs by certain serum proteins and their physiological action is expressed by binding to steroid receptors.[3] These steroids may be converted to bioactive substances or degraded by the action of various enzymes.[4] Some steroids act as regulatory factors which directly activate or inhibit the enzymes involved in steroid metabolism.[5] Therefore, studies on the molecular interactions between steroids and proteins are essential for an understanding of the molecular basis of the biosynthesis, degradation, and physiological action of these steroids. In recent years, much interest has centered upon the structural features of steroid molecules which permit recognition

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and functional interaction to occur.[6] However, it is not always easy to obtain those bioactive proteins in quantity and in a pure form, and it is sometimes difficult to detect subtle differences in the interactions, caused by minor structural changes in the steroid molecule. In our work, 20β-hydroxysteroid dehydrogenase [EC 1.1.1.53] from *Streptomyces hydrogenans* was used as a model protein, because this enzyme is commercially available as a pure crystalline enzyme in sufficient quantity for detailed comparative studies of steroid-protein interactions with various substituted steroids. The enzyme, which transfers hydrogen from NADH to various 20-oxo steroids to give 20β-hydroxysteroids, is thought to be sensitive to structural changes in the region around C-20, involving C-16, C-17, and C-21 of the steroid. It would also be expected to respond to structural changes in other parts of the steroid molecule, which may influence the interactions of the steroid with the enzyme protein directly or indirectly. Since the biological functions of steroid hormones are at least partly dependent upon the structural features around C-17 or the C-17 β-side chain, a comparative investigation of a particular system might yield results of general interest. In this paper, the substrate specificity and kinetic properties of 20β-hydroxysteroid dehydrogenase with various substituted steroids are described.

Experimental

Materials——The 20-oxo steroids listed in Table I were used in this study. Most of the steroids were purchased from Sigma Chemical Co., E. Merck AG or Fluka AG. Each steroid was of the purest grade commercially obtainable. Progesterone, hydrocortisone, prednisolone, cortisone acetate, hydrocortisone acetate, and prednisolone acetate were standard substances from the National Institute of Hygienic Sciences, Tokyo. Fluorometholone was kindly supplied by Kowa Co., Ltd., Tokyo. Fluocortolone was from Nihon Schering Co., Ltd., Osaka. Betamethasone 17-valerate and beclomethasone dipropionate were from Fuji Yakuhin Laboratory, Tokyo. Betamethasone acetate was from Shionogi Co., Ltd., Osaka. The purity of the steroids supplied was examined by high-performance liquid chromatography as described below, and no impurity was detectable under the conditions used. NADH was purchased from Sigma Chemical Co. and Oriental Yeast Co., Ltd., Tokyo. The 20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* was obtained from Boehringer Mannheim Co. The enzyme preparation was a highly purified crystalline material, and gave one protein band on polyacrylamide gel electrophoresis. The purity of the enzyme was further examined as follows: 20β-hydroxypregn-4-en-3-one, 20α-hydroxypregn-4-en-3-one, $17\alpha,20\alpha,21$-trihydroxypregn-4-ene-3,11-dione, 4-androstene-3,17-dione and 4-androstene-3,11,17-trione (each 66.7 μM) were each incubated with the enzyme (12 μg/3 ml) in 0.1 M phosphate buffer containing 1 mM EDTA, pH 6.4, at 25° in the presence of NADH (150 μM). No loss of NADH was detected under these conditions, where $11\beta,17\alpha,21$-trihydroxypregn-4-ene-3,20-dione, which is a relatively poor substrate (see the relative rate under Compd. No. IB-7 in Table II), reacted rapidly; this indicated that this enzyme preparation catalyzed the reduction of the 20-oxo group only and contained no activity for the reduction of the Δ^4, 3-oxo, 11-oxo or 17-oxo group. In the presence of NAD (300 μM) and the enzyme (0.8 μg/3 ml), 20β-hydroxypregn-4-en-3-one (66.7 μM) reacted briefly in 0.1 M Tris buffer containing 1 mM EDTA, pH 8.75, at 25°, but no reaction was detected with 20α-hydroxypregn-4-en-3-one (66.7 μM), 5β-pregnane-$3\alpha,20\alpha$-diol (22.2 μM), $17\alpha,20\alpha,21$-trihydroxypregn-4-ene-3,11-dione (66.7 μM), 5α-androstane-$3\beta,17\beta$-diol (44.5 μM) or $11\beta,17\alpha,21$-trihydroxypregn-4-ene-3,20-dione (66.7 μM) in the presence of NAD with 5—15 times the amounts of the enzyme. Thus, this enzyme preparation had 20β-hydroxysteroid dehydrogenase activity, but contained no 20α-, 17α-, 17β-, 11β-, 3α- or 3β-hydroxysteroid dehydrogenase activity; indeed, with pregn-4-ene-3,20-dione it was established by high-performance liquid chromatography that the sole product of enzymatic reaction was 20β-hydroxypregn-4-en-3-one. When 17-hydroxypregn-4-ene-3,20-dione was used as a substrate the sole reaction product showed the same retention time as $17,20\beta$-dihydroxypregn-4-en-3-one, on the chromatograms, and did not coincide with 4-androstene-3,17-dione.

High-Performance Liquid Chromatography——A Hitachi 635 liquid chromatograph was used with a double beam grating spectrophotometer operated at a wavelength of 241 nm (range: 0.16 O.D.). The stainless steel column (150 mm $\times$ 4 mm I.D.) was packed with Lichrosorb RP-18 (E. Merck; mean particle size 5 μm). The column was operated at about 20° and at a flow rate of 0.54 ml/min (pressure: 60—120 kg/cm^2). The mobile phase was a mixture of analytical grade acetonitrile and distilled water in various ratios (6: 4, 5: 5, 4: 6, 3: 6, 3: 7 or 1: 3). Steroids dissolved in methanol (1 μg in 1—3 μl) were injected into the column.

6) I.H. White and J. Jeffery, *Eur. J. Biochem.*, **25**, 409 (1972); *idem, Biochim. Biophys. Acta*, **296**, 604 (1973); *idem, Biochem. J.*, **137**, 349 (1974); J.C. Warren, "Advan. Exp. Med. Biol.," Vol. 36, ed. by B.W. O'Malley and A.R. Means, Plenum Press, 1973, p. 263; H.E. Smith, R.G. Smith, D.O. Toft, J.R. Neergaard, E.P. Burrows and B.W. O'Malley, *J. Biol. Chem.*, **249**, 5924 (1974).

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Table I. Steroids used as Test Substrates

Compd. No.	Systematic name (trivial name)
IA-1	Pregn-4-ene-3,20-dione (progesterone)
IA-2	17-Hydroxypregn-4-ene-3,20-dione (17α-hydroxyprogesterone)
IA-3	Pregn-4-ene-3,11,20-trione (11-ketoprogesterone)
IA-4	17-Hydroxypregn-4-ene-3,11,20-trione (21-deoxycortisone)
IA-5	17-Hydroxy-6α-methylpregn-4-ene-3,20-dione (6α-methyl-17α-hydroxyprogesterone)
IA-6	11α-(Acetyloxy)pregn-4-ene-3,20-dione (11α-acetoxyprogesterone)
IA-7	11α-Hydroxypregn-4-ene-3,20-dione (11α-hydroxyprogesterone)
IA-8	11β-Hydroxypregn-4-ene-3,20-dione (11β-hydroxyprogesterone)
IA-9	18-Hydroxypregn-4-ene-3,20-dione (18-hydroxyprogesterone)
IA-10	Pregna-4,16-diene-3,20-dione
IA-11	6α-Methylpregna-4,16-diene-3,20-dione
IA-12	16α-Methylpregn-4-ene-3,20-dione (16α-methylprogesterone)
IA-13	16β-Methylpregn-4-ene-3,20-dione (16β-methylprogesterone)
IA-14	17-(Acetyloxy)pregn-4-ene-3,20-dione (17α-acetoxyprogesterone)
IA-15	17-[(1-oxo-hexyl)oxy]pregn-4-ene-3,20-dione (17α-hydroxyprogesterone caproate)
IB-1	21-Hydroxypregn-4-ene-3,20-dione (deoxycorticosterone)
IB-2	17,21-Dihydroxypregn-4-ene-3,20-dione (11-deoxycortisol)
IB-3	21-Hydroxypregn-4-ene-3,11,20-trione (11-dehydrocorticosterone)
IB-4	17,21-Dihydroxypregn-4-ene-3,11,20-trione (cortisone)
IB-5	6β,17,21-Trihydroxypregn-4-ene-3,11,20-trione (6β-hydroxycortisone)
IB-6	11β,21-Dihydroxypregn-4-ene-3,20-dione (corticosterone)
IB-7	11β,17,21-Trihydroxypregn-4-ene-3,20-dione (hydrocortisone)
IB-8	11β,21-Dihydroxy-3,20-dioxopregn-4-en-18-al (aldosterone)
IB-9	21-(Acetyloxy)pregn-4-ene-3,20-dione (deoxycorticosterone acetate)
IB-10	21-(Acetyloxy)-17-hydroxypregn-4-ene-3,11,20-trione (cortisone acetate)
IB-11	21-(Acetyloxy)-11β,17-dihydroxypregn-4-ene-3,20-dione (hydrocortisone acetate)
IB-12	21-(Phosphonooxy)-11β,17-dihydroxypregn-4-ene-3,20-dione, disodium salt (hydrocortisone phosphate sodium)
IIA-1	5α-Pregnane-3,20-dione
IIA-2	3β-Hydroxy-5α-pregnan-20-one
IIA-3	3β-(Acetyloxy)-5α-pregnan-20-one
IIA-4	5α-Pregnane-3,11,20-trione
IIB-1	17,21-Dihydroxy-5α-pregnane-3,11,20-trione (allodihydrocortisone)
IIB-2	3β,17,21-Trihydroxy-5α-pregnan-20-one
IIIA-1	5β-Pregnane-3,20-dione
IIIA-2	3α-Hydroxy-5β-pregnan-20-one
IIIA-3	3β-Hydroxy-5β-pregnan-20-one
IIIA-4	3α,6α-Dihydroxy-5β-pregnan-20-one
IIIA-5	3α,17-Dihydroxy-5β-pregnan-20-one
IIIA-6	3α-Hydroxy-5β-pregnane-11,20-dione
IIIA-7	3α,17-Dihydroxy-5β-pregnane-11,20-dione
IIIA-8	3α,7α,12α-Trihydroxy-5β-pregnan-20-one
IIIB-1	3α,17,21-Trihydroxy-5β-pregnan-20-one
IIIB-2	3α,17,21-Trihydroxy-5β-pregnane-11,20-dione (tetrahydrocortisone)
IIIB-3	3α,11β,21-Trihydroxy-5β-pregnan-20-one (tetrahydrocorticosterone)
IIIB-4	3α,11β,17,21-Tetrahydroxy-5β-pregnan-20-one (tetrahydrocortisol)
IVA-1	3β-Hydroxypregn-5-en-20-one (pregnenolone)
IVA-2	3β-Hydroxy-6-methylpregn-5-en-20-one (6-methylpregnenolone)
IVA-3	3β,17-Dihydroxypregn-5-en-20-one (17α-hydroxypregnenolone)
IVA-4	3β,17-Dihydroxy-6-methylpregn-5-en-20-one (6-methyl-17α-hydroxypregnenolone)
IVB-1	21-(Acetyloxy)-3β-hydroxypregn-5-en-20-one (21-acetoxypregnenolone)
VB-1	17,21-Dihydroxypregna-1,4-diene-3,11,20-trione (prednisone)
VB-2	11β,17,21-Trihydroxypregna-1,4-diene-3,20-dione (prednisolone)
VB-3	11β,17,21-Trihydroxy-6α-methylpregna-1,4-diene-3,20-dione (6α-methylprednisolone)
VB-4	6α-Fluoro-11β,21-dihydroxy-16α-methylpregna-1,4-diene-3,20-dione (fluocortolone)
VB-5	9-Fluoro-11β,17-dihydroxy-6α-methylpregna-1,4-diene-3,20-dione (fluorometholone)
VB-6	9-Fluoro-11β,16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione (triamcinolone)
VB-7	9-Fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione (dexamethasone)

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Compd. No.	Systematic name (trivial name)
VB-8	9-Fluoro-11β,17,21-trihydroxy-16β-methylpregna-1,4-diene-3,20-dione (betamethasone)
VB-9	9-Fluoro-11β,21-dihydroxy-17-valeryloxy-16β-methylpregna-1,4-diene-3,20-dione (betamethasone 17-valerate)
VB-10	9-Fluoro-11β,21-dihydroxy-16α,17-[(1-methylethylidene)bis(oxy)]pregna-1,4-diene-3,20-dione (triamcinolone acetonide)
VB-11	6α,9-Difluoro-11β,21-dihydroxy-16α,17-[(1-methylethylidene)bis(oxy)]pregna-1,4-diene-3,20-dione (fluocinolone acetonide)
VB-12	9-Chloro-11β-hydroxy-16β-methyl-17,21-bis(1-oxopropoxy)-pregna-1,4-diene-3,20-dione (beclomethasone dipropionate)
VB-13	21-(Acetyloxy)-11β,17-dihydroxypregna-1,4-diene-3,20-dione (prednisolone 21-acetate)
VB-14	21-(Acetyloxy)-9-fluoro-11β,17-dihydroxy-16β-methylpregna-1,4-diene-3,20-dione (betamethasone 21-acetate)

Characterization of Enzymic Reaction Products——The reaction products of pregn-4-ene-3,20-dione (IA-1) and 17-hydroxypregn-4-ene-3,20-dione (IA-2) were prepared as follows. The reaction mixtures, containing 1 μmol of steroid, 4 μmol of NADH, 0.4 ml of methanol, 0.2 mg of the enzyme, 0.6 mmol of sodium phosphate buffer (pH 6.4) and 6 μmol of EDTA in a final volume of 5.9 ml, were incubated at 25°. After 25 min, 1 μmol of NADH (50 μl) and appropriate amounts of the enzyme [0.2 mg (0.1 ml) in the case of IA-2; 0.1 mg (50 μl) in the case of IA-1] were added to the mixture and the reaction was continued for a further 25 min. The reaction mixture was then extracted with dichloromethane (3×30 ml). The combined extracts were washed with water (40 ml), dried with Na_2SO_4, filtered, and evaporated to dryness on a rotary evaporator at room temperature. The residue was dissolved in 0.35 ml of methanol and aliquots (0.6—1.3 μl) were examined by high-performance liquid chromatography using a mixture of acetonitrile and distilled water in a ratio of 5: 5 as the mobile phase. Control runs without NADH or enzyme in the reaction mixtures were carried out in the same manner. Authentic samples of 20β-hydroxypregn-4-en-3-one, 20α-hydroxypregn-4-en-3-one, 17,20β-dihydroxypregn-4-en-3-one or 4-androstene-3,17-dione were used as chromatographic standards.

Assay of 20β-Hydroxysteroid Dehydrogenase Activity——The enzyme activity was assayed spectrophotometrically at 25° and initial velocities were determined by measuring the decrease in absorption of NADH at 340 nm with a Union High-Sens. SM-401 spectrophotometer equipped with a temperature-controlled cuvette chamber and a National X-Y recorder. The reaction mixture contained, in a final volume of 3 ml, the following components (μmol): sodium phosphate buffer (pH 6.4), 300; EDTA, 3; NADH, 0.45; steroids, 0.06—0.2; and appropriate amounts of the enzyme. Steroids were dissolved in methanol. The concentration of methanol was kept below 10% to avoid inhibition of the enzyme activity. The reaction was initiated by addition of the enzyme. The initial rate of reaction was determined from the linear part of the reaction curve (the first 10—20%). The amount of enzyme used was chosen so that the reaction was about 10—20% complete in about 1—3 min. For steroids having a V value (μmol/min/mg of enzyme) of above 10 (good substrates), 1—1.25 μg of enzyme was used; for steroids having V values of 2—10 and 1—0.1, 2—10 μg and 20—25 μg of enzyme were used, respectively; for very poor or inactive substrate steroids, 50—100 μg of enzyme was used.

The linear regression plots for the reciprocal of the initial reaction rate against the reciprocal of the substrate concentration were calculated with a computer using the programs of Cleland.[7] Standard errors of the apparent K_m and apparent V_{max} were estimated by Wilkinson's method.[8]

The optimum pH of the enzyme was determined in 0.1 M sodium phosphate buffer of various pH's with progesterone (pregn-4-ene-3,20-dione) and cortisone (17,21-dihydroxypregn-4-ene-3,11,20-trione) as substrates. The optimum pH was 6.4 in both cases.

Concentrations of the Enzyme, Coenzyme and Steroids——The concentration of 20β-hydroxysteroid dehydrogenase was determined from its specific activity, which was 18.65 μmol/min/mg of enzyme when assayed in 0.1 M sodium phosphate buffer (pH 6.4) containing 1 mM EDTA, 0.15 mM NADH, 0.46 mM cortisone and 10% methanol at 25°. In some cases, 0.2 mM hydrocortisone (11β,17,21-trihydroxypregn-4-ene-3,20-dione) was used as a positive control in place of cortisone, and in this case the specific activity of the enzyme was 1.38 μmol/min/mg of enzyme. NADH concentration was determined from the absorbance at 340 nm, using a molar extinction coefficient of $6.2\times10^3\,M^{-1}\cdot cm^{-1}$. The concentration of steroids were based upon weights of dried samples.

7) W.W. Cleland, *Nature* (London), **198**, 463 (1963).
8) G.N. Wilkinson, *Biochem. J.*, **80**, 324 (1961).

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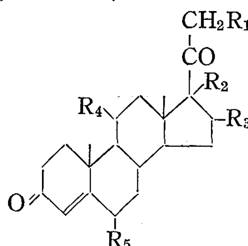
Steroid Solubility——A fixed volume (0.3 ml) of steroid dissolved in methanol was added to 2.7 ml of sodium phosphate buffer in a cuvette of 10 mm light pathway. Using careful visual inspection or absorbance measurement, the highest steroid concentration was determined at which precipitation was detected within 10 min of mixing. If precipitation, probably due to the reaction product, was detected during the enzymatic reaction, the reaction rate was reexamined with a smaller amount of steroid. Among the steroids, IA-15, IIA-1, IIA-2, IIA-3, IVB-1 and VB-12 came out of solution before or during the reaction when used in amounts of over 0.06 or 0.08 μmol, and IA-13, IIIA-2, IVA-1, IVA-2, IVA-3 and IVA-4 did so when used in amounts of over 0.1 or 0.15 μmol. No precipitation was detected with over 0.2 μmol of all the other steroids.

Results and Discussion

Substrate Specificity of 20β-Hydroxysteroid Dehydrogenase

The reaction rates of 64 steroids relative to that of progesterone are shown in Tables II—VI. The rate of reduction of pregnan-20-one derivatives was influenced by substituents at various positions of the steroid skeleton.

Table II. Substrate Specificity of 20β-Hydroxysteroid Dehydrogenase for Pregn-4-ene-3,20-dione Derivatives



Compd. No.	R_1 (C-21)	R_2 (C-17α)	R_3 (C-16)	R_4 (C-11)	R_5 (C-6)	Other	V (μmol/min/mg)	Relative rate (%)
IA-1	H	H	H	H	H		22.27	100
IA-2	H	OH	H	H	H		9.32	41.9
IA-3	H	H	H	=O	H		29.52	132.6
IA-4	H	OH	H	=O	H		28.75	129.1
IA-5	H	OH	H	H	α-CH_3		4.33	19.4
IA-6	H	H	H	α-$OCOCH_3$	H		5.03	22.6
IA-7	H	H	H	α-OH	H		2.07	9.3
IA-8	H	H	H	β-OH	H		1.22	5.5
IA-9	H	H	H	H	H	18-OH	0.55	2.5
IA-10	H	Δ^{16}		H	H		0.22	1.0
IA-11	H	Δ^{16}		H	α-CH_3		0.21	1.0
IA-12	H	H	α-CH_3	H	H		0.06	0.3
IA-13	H	H	β-CH_3	H	H		0[a]	0
IA-14	H	$OCOCH_3$	H	H	H		0	0
IA-15	H	$OCO(CH_2)_4CH_3$	H	H	H		0[b]	0
IB-1	OH	H	H	H	H		3.39	15.2
IB-2	OH	OH	H	H	H		9.07	40.7
IB-3	OH	H	H	=O	H		2.42	10.9
IB-4	OH	OH	H	=O	H		9.60	43.1
IB-5	OH	OH	H	=O	β-OH		0.61	2.8
IB-6	OH	H	H	β-OH	H		0.14	0.6
IB-7	OH	OH	H	β-OH	H		0.57	2.6
IB-8	OH	H	H	β-OH	H	18-CHO	0.01	0.0
IB-9	$OCOCH_3$	H	H	H	H		0.02	0.1
IB-10	$OCOCH_3$	OH	H	=O	H		0	0
IB-11	$OCOCH_3$	OH	H	β-OH	H		0	0
IB-12	OPO_3Na_2	OH	H	β-OH	H		0	0

The amount of substrate steroid used was 0.2 μmol, except that *a*) and *b*) were 0.1 and 0.06 μmol, respectively.
The rates for the different steroids are given relative to that of pregn-4-ene-3,20-dione taken as 100%.

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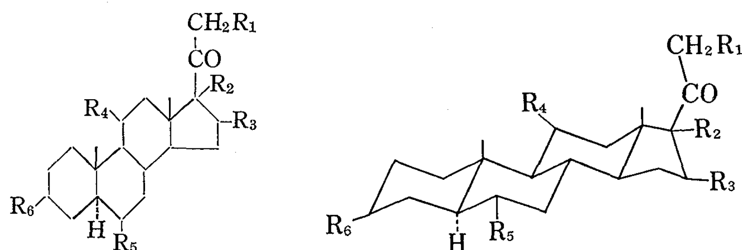
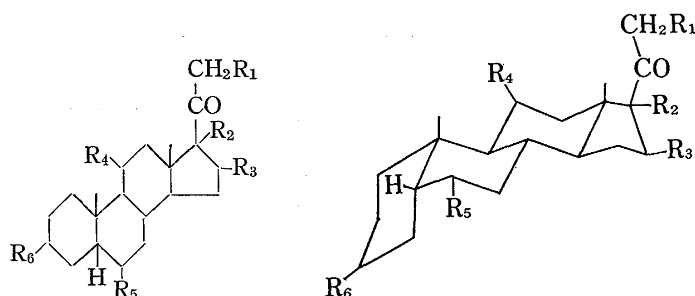
TABLE III. Substrate Specificity of 20β-Hydroxysteroid Dehydrogenase for 5α-Pregnan-20-one Derivatives



Compd. No.	Substrate R$_1$ (C-21)	R$_2$ (C-17α)	R$_3$ (C-16)	R$_4$ (C-11)	R$_5$ (C-6)	R$_6$ (C-3)	V (μmol/min/mg)	Relative rate (%)
IIA-1	H	H	H	H	H	=O	13.70[a]	61.5
IIA-2	H	H	H	H	H	β-OH	8.47[a]	38.0
IIA-3	H	H	H	H	H	β-OCOCH$_3$	4.20[a]	18.8
IIA-4	H	H	H	=O	H	=O	17.27	77.5
IIB-1	OH	OH	H	=O	H	=O	4.74	21.3
IIB-2	OH	OH	H	H	H	β-OH	4.85	21.8

The amount of substrate steroid used was 0.2 μmol, except that *a*) was 0.06 μmol. The rates are given relative to that of pregn-4-ene-3,20-dione taken as 100%.

TABLE IV. Substrate Specificity of 20β-Hydroxysteroid Dehydrogenase for 5β-Pregnan-20-one Derivatives



Compd. No.	Substrate R$_1$ (C-21)	R$_2$ (C-17α)	R$_3$ (C-16)	R$_4$ (C-11)	R$_5$ (C-6)	R$_6$ (C-3)	Other	V (μmol/min/mg)	Relative rate (%)
IIIA-1	H	H	H	H	H	=O		9.95	44.7
IIIA-2	H	H	H	H	H	α-OH		5.67[a]	25.4
IIIA-3	H	H	H	H	H	β-OH		5.68	25.5
IIIA-4	H	H	H	H	α-OH	α-OH		6.29	28.3
IIIA-5	H	OH	H	H	H	α-OH		3.62	16.2
IIIA-6	H	H	H	=O	H	α-OH		3.90	17.5
IIIA-7	H	OH	H	=O	H	α-OH		14.65	65.8
IIIA-8	H	H	H	H	H	α-OH	7α-OH 12α-OH	0.08	0.4
IIIB-1	OH	OH	H	H	H	α-OH		4.38	19.7
IIIB-2	OH	OH	H	=O	H	α-OH		2.81	12.6
IIIB-3	OH	H	H	β-OH	H	α-OH		0.03	0.1
IIIB-4	OH	OH	H	β-OH	H	α-OH		0.32	1.4

The amount of substrate steroid used was 0.2 μmol, except that *a*) was 0.1 μmol. The rates are given relative to that of pregn-4-ene-3,20-dione taken as 100%.

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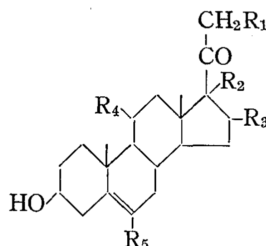
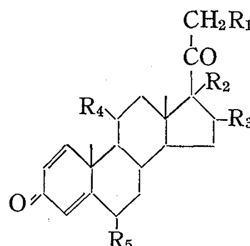
Table V. Substrate Specificity of 20β-Hydroxysteroid Dehydrogenase for 3β-Hydroxypregn-5-en-20-one Derivatives



Compd. No.	Substrate R_1 (C-21)	R_2 (C-17α)	R_3 (C-16)	R_4 (C-11)	R_5 (C-6)	V (μmol/min/mg)	Relative rate (%)
IVA-1	H	H	H	H	H	7.14	32.1
IVA-2	H	H	H	H	CH_3	5.87	26.3
IVA-3	H	OH	H	H	H	5.14	23.1
IVA-4	H	OH	H	H	CH_3	4.38	19.7
IVB-1	$OCOCH_3$	H	H	H	H	0.01[a]	0.0

The amount of substrate steroid used was 0.1 μmol, except that *a*) was 0.06 μmol. The rates are given relative to that of pregn-4-ene-3,20-dione taken as 100%.

Table VI. Substrate Specificity of 20β-Hydroxysteroid Dehydrogenase for 21-Hydroxypregna-1,4-diene-3,20-dione



Compd. No.	Substrate R_1 (C-21)	R_2 (C-17α)	R_3 (C-16)	R_4 (C-11)	R_5 (C-6)	Other	V (μmol/min/mg)	Relative rate (%)
VB-1	OH	OH	H	=O	H		12.94	58.1
VB-2	OH	OH	H	β-OH	H		0.46	2.1
VB-3	OH	OH	H	β-OH	α-CH_3		0.78	3.5
VB-4	OH	H	α-CH_3	β-OH	α-F		0	0
VB-5	H	OH	H	β-OH	α-CH_3	9-F	4.70	21.1
VB-6	OH	OH	α-OH	β-OH	H	9-F	0.02	0.1
VB-7	OH	OH	α-CH_3	β-OH	H	9-F	0	0
VB-8	OH	OH	β-CH_3	β-OH	H	9-F	0	0
VB-9	OH	$OCO(CH_2)_3CH_3$	β-CH_3	β-OH	H	9-F	0	0
VB-10	OH	O–C(H_3C)(CH_3)–O (spanning R_2 and R_3)		β-OH	H	9-F	0	0
VB-11	OH	O–C(H_3C)(CH_3)–O (spanning R_2 and R_3)		β-OH	α-F	9-F	0	0
VB-12	$OCOCH_2CH_3$	$OCOCH_2CH_3$	β-CH_3	β-OH	H	9-Cl	0[a]	0
VB-13	$OCOCH_3$	OH	H	β-OH	H		0	0
VB-14	$OCOCH_3$	OH	β-CH_3	β-OH	H	9-F	0	0

The amount of substrate steroid used was 0.2 μmol, except that *a*) was 0.06 μmol. The rates are given relative to that of pregn-4-ene-3,20-dione taken as 100%.

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The steroids esterified at the C-17 or C-21 hydroxyl group with acetate, caproate or phosphate did not serve as substrates (IA-14, IA-15, IB-9, IB-10, IB-11, and IB-12 in Table II, IVB-1 in Table V, VB-13 in Table VI). In contrast, steroids esterified at the C-3 or C-11 hydroxyl group were utilized at about 20% of the reaction rate of progesterone (IIA-3 in Table III and IA-6 in Table II). This seems to be attributable to the existence of a bulky substituent adjacent to the reacting 20-oxo group; such a bulky substituent may inhibit the proper interaction of the 20-oxo group with the catalytic site of the enzyme. The introduction of a methyl group at the C-16 β-position (IA-13 in Table II and VB-8 in Table VI) resulted in loss of reactivity. The existence of a methyl or hydroxyl group at the α-position of C-16 markedly reduced the reaction rate to less than 0.3% of that for progesterone or abolished the reactivity (IA-12 in Table II and VB-4, VB-6, and VB-7 in Table VI). This suggests that these small substituents have an unfavorable effect on the catalytic reaction. 16α,17α-Acetonide derivatives (VB-10 and VB-11 in Table VI) did not act as substrates. Steroids having both a 16β-methyl group and a bulky group at C-21 or C-17 (VB-9, VB-12, and VB-14 in Table VI) were, of course, not substrates. The 18-hydroxyl derivative (IA-9 in Table II) was a poor substrate, and 11β,21-dihydroxy-3,20-dioxopregn-4-en-18-al (IB-8), which contained an aldehyde group in place of the methyl group at C-18, was also a very poor substrate. Introduction of a double bond into the D-ring reduced the reaction rate to less than 1% of the rate for progesterone (IA-10 and IA-11 in Table II), although introduction of a double bond in the A- or B-ring did not significantly influence the reaction rate (IVA-1, IVA-2, and IVA-3 in Table V and VB-1 in Table VI). This decrease of the rate may be caused by a marked change in the configuration of the C-17 side chain upon the introduction of a C-16—C-17 double bond into the D-ring, so that the C-17—C-20 chain and D-ring become nearly coplanar. Introduction of a hydroxyl group into C-11 greatly decreased the reaction rate, and the rates for 11-hydroxyl derivatives were less than 10% of that for progesterone (IA-7 and IA-8 in Table II). The presence of a 21-hydroxyl (*e.g* IB-1, IB-2, IB-3, and IB-4), 17α-hydroxyl (*e.g.* IA-2), 11-oxo (*e.g.* IA-3), 11-acetyloxy (*e.g.* IA-6), 9-fluoro (*e.g.* VB-5), 6-methyl (*e.g.* IA-5), 6α-hydroxyl (*e.g.* IIIA-4), 3-hydroxyl (*e.g.* IIA-2) or 3-acetyloxy (*e.g.* IIA-3) group on 20-oxo steroid derivatives, saturation of the C-4—C-5 double bond (*e.g.* IIA-1 and IIIA-1) and the introduction of a C-5—C-6 double bond (*e.g.* IVA-1) or double bonds at both C-1—C-2 and C-4—C-5 (*e.g.* VB-1) did not cause the reaction rate to fall below 10% of that for the parent compound, progesterone (Tables II—VI).

These results suggest that the presence and nature of a substituent around the reacting 20-oxo group of the steroid markedly affect the interaction with the catalytic site of the enzyme. The configurational relationship between the plane of the steroid ring and the side chain of C-17 may also be important. Substituents at other regions of the steroid molecule had various effects on the reactivity in the enzyme reaction, but these were relatively minor. Evaluation of these results may provide information on the steroid-enzyme interaction, especially regarding the binding site on the steroid molecule involved in the binding process with the binding site on the enzyme.

Inhibitory Effects of 16β-Methylpregn-4-ene-3,20-dione on the Enzymic Reaction

Steroids having a bulky group near the reacting 20-oxo group and substituent at C-16 were not reactive. It is noteworthy that even a small substituent such as a methyl or hydroxyl group at C-16 led to loss of reactivity as a substrate, whereas the introduction of a 17α-hydroxyl group did not necessarily reduce the reactivity. A small substituent at C-16, especially in the α-position, is spatially further from the reacting 20-oxo group than a 17α-hydroxyl group. Therefore, it seems likely that the mechanism of loss of reactivity caused by a small substituent at C-16 may be different from that in the case of a bulky substituent at C-17 or C-21.

To examine this, the enzymic reaction was carried out with 66.7 μM 17,21-dihydroxypregn-4-ene-3,11,20-trione (cortisone) as a substrate in the presence of cortisone 21-acetate or 16β-methylpregn-4-ene-3,20-dione. Cortisone 21-acetate (66.7 μM) had no effect on the reduction

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of cortisone, but 33.3 μM and 66.7 μM 16β-methylpregn-4-ene-3,20-dione inhibited the reaction by 46 and 66%, respectively. This suggests that owing to the bulky substituent at C-21, cortisone 21-acetate had lost the ability to interact with the enzyme. On the other hand, 16β-methylpregn-4-ene-3,20-dione may be able to interact with the enzyme, but the 16β-methyl group appears to have an unfavorable effect on the catalytic process.

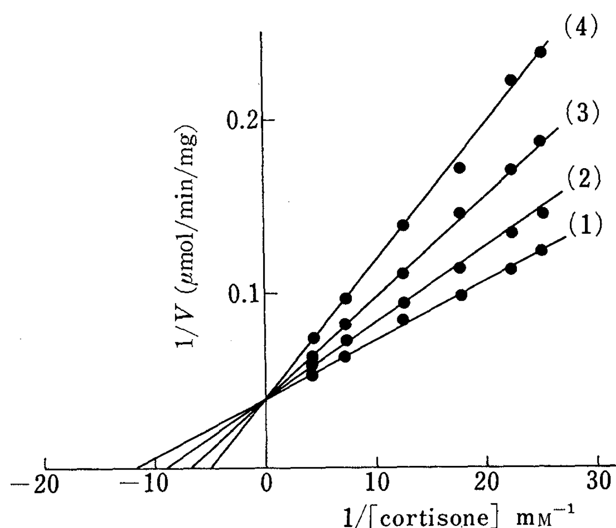

Fig. 1. Inhibition of 20β-Hydroxysteroid Dehydrogenase Activity by 16β-Methylpregn-4-ene-3,20-dione with Cortisone as the Substrate

The concentrations of 16β-methylpregn-4-ene-3,20-dione were: 1, 0 μM; 2, 12 μM; 3, 24 μM; 4, 30 μM.

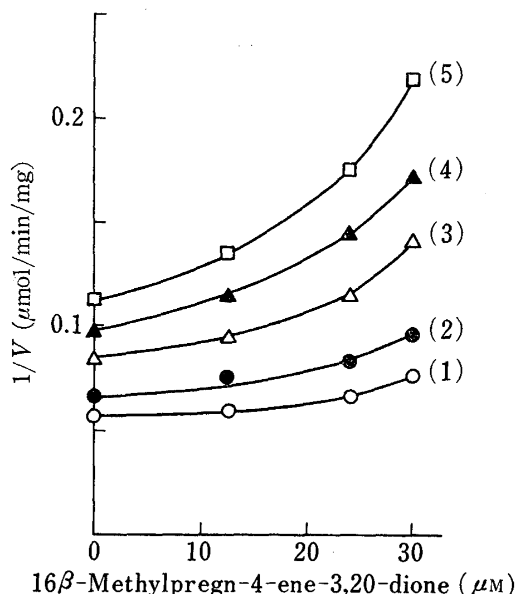

Fig. 2. Dixon Plots of the Data from Fig. 1

The concentrations of cortisone were: 1, 138 μM; 2, 80 μM; 3, 57 μM; 4, 45 μM; 5, 40 μM.

To obtain further information, kinetic analyses of the reaction of the 16-methyl derivative were carried out. When initial velocity measurements were performed with cortisone as a variable substrate (40—229 μM), NADH as a fixed substrate (150 μM) and 16β-methylpregn-4-ene-3,20-dione as an inhibitor and the data were plotted by the method of Lineweaver and Burk,[9] a competitive pattern was observed, as shown in Fig. 1. The apparent K_m of cortisone increased from 76 μM to 185 μM as the concentration (0—30 μM) of the inhibitor was varied. However, Dixon plots[10] of the inhibition of the enzyme by 16β-methylpregn-4-ene-3,20-dione were nonlinear (Fig. 2). The reason for the nonlinearity is not clear, but it seems likely that the 16-methyl derivative may inhibit not merely the substrate binding process but also the hydrogen transfer process involving the coenzyme. If this is the case, the 16-methyl derivative may also be an inhibitor with respect to the coenzyme and this may account for the competitive nature of the inhibition. With NADH as a variable substrate (37—150 μM), cortisone as a fixed substrate (460 μM) and 16β-methylpregn-4-ene-3,20-dione as an inhibitor, the initial velocity of the reaction were determined. As shown in Table VII, there was little significant variation in the apparent V_{max}, whereas the apparent K_m increased with increasing concentration of the inhibitor. This indicates that the type of inhibition is competitive.

The finding that 16β-methylpregn-4-ene-3,20-dione is not a substrate, but a competitive inhibitor for both steroid and coenzyme indicated that there are two regions of the inhibitor molecule, one of which interacts with the substrate binding site of the enzyme and another competes with the coenzyme action. Since pregn-4-ene-3,20-dione is a good substrate and

9) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).
10) M. Dixon, *Biochem. J.*, **55**, 170 (1953).

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Table VII. Effect of 16β-Methylpregn-4-ene-3,20-dione on the Kinetic Parameters of 20β-Hydroxysteroid Dehydrogenase for NADH

Concentration of 16β-methylpregn-4-ene-3,20-dione (μM)	Apparent K_m (μM)	Apparent V_{max} (μmol/min/mg)
0	3.34±0.50	19.56±0.15
18	7.05±0.75	19.09±0.21
30	9.32±1.16	18.30±0.30

Cortisone (460 μM) was used as a fixed substrate.

has a relatively low apparent K_m value (4.49±0.04 μM), the region which competes with the substrate binding process may be assigned to the steroid ring and that which inhibits the coenzyme action may be assigned to the 16β-methyl substituent.

The Apparent K_m for NADH with 16α-Methylpregn-4-ene-3,20-dione as a Substrate

It has been shown that the reaction mechanism of 20β-hydroxysteroid dehydrogenase is essentially an ordered Bi Bi mechanism, in which the enzyme first binds the coenzyme and then the steroid, hydrogen is transferred, and the steroid product leaves the enzyme followed by the coenzyme product.[11] Taking this view, the apparent K_m for NADH should be independent of the steroid substrate. However, if a steroid substrate affects the coenzyme-enzyme interaction in the binary or ternary complex, the apparent K_m for NADH will not be independent of the steroid substrate.

It has been suggested that the presence of a substituent at C-16 of the steroid ring affects the coenzyme action. Therefore, using 16α-methylpregn-4-ene-3,20-dione, a poor substrate (Table II), the kinetic parameters for NADH were determined and compared with those obtained using pregn-4-ene-3,20-dione and 17,21-dihydroxypregn-4-ene-3,11,20-trione as the fixed substrate. As shown in Table VIII, the substituent at C-16 unfavorably affected the apparent K_m for NADH but those at C-21, C-17, or C-11 did not.

Table VIII. The Apparent K_m of 20β-Hydroxysteroid Dehydrogenase for NADH using Several 20-Oxo Steroids as Substrates

Fixed substrate (concentration)	Apparent K_m for NADH (μM)
Pregn-4-ene-3,20-dione (165 μM)	3.91±0.77
17,21-Dihydroxypregn-4-ene-3,11,20-trione (460 μM)	4.07±0.65
16α-Methylpregn-4-ene-3,20-dione (250 μM)	13.97±1.06

Coupled with the results of the inhibition experiment with the 16β-methyl derivative, these findings suggest that the coenzyme may be located in the vicinity of C-16 of the steroid molecule (probably on the β-side) in the ternary complex, steroid-coenzyme-enzyme, during the catalytic process, and the existence of the substituent at C-16 may inhibit the coenzyme interaction with the enzyme protein as well as with the reacting 20-oxo group.

In summary, the binding interaction with the enzyme may involve the steroid ring, while the catalytic process involves the region around C-17—C-21, centering on C-20. The coenzyme may be located in the vicinity of C-16 in the ternary complex. Further studies are required on the interactions at other regions of the steroid molecule.

11) G. Betz and J.C. Warren, *Arch. Biochem. Biophys.*, **128**, 745 (1968); G. Betz and P. Taylor, *ibid.*, **137**, 109 (1970).

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