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Structural Requirements in 20-Oxo-steroids for Interaction with the Catalytic Site of 20β-Hydroxysteroid Dehydrogenase

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Kinetic measurements were made to investigate the interaction of 20β-hydroxysteroid dehydrogenase and a series of steroids with C-17 and/or C-21 hydroxyl groups, and the role of the region around C-17 and C-21 on the interaction of the reacting 20-oxo group with the catalytic site of the enzyme was considered. Substitution of a hydroxyl group for hydrogen at C-21 of 17-deoxy-steroid derivatives caused a significant decrease in the apparent V_{max} value (to about one-sixth to one-ninth), but a slight increase in the apparent K_m value (about 1.1- to 1.8-fold). The same change at C-21 of 17-hydroxy-steroid derivatives caused little or no decrease in the apparent V_{max} value, but an increase in the apparent K_m (about 1.4- to 1.8-fold) occurred which was similar to that with 17-deoxy-steroid derivatives. In 21-deoxy-11-deoxy-steroid derivatives, a 17α-hydroxyl substituent had little effect on the apparent K_m value (about 0.8- to 1.1-fold) and produced a slight decrease in the apparent V_{max} value (to about three-quarters to two-fifths). Introduction of a 17α-hydroxyl group into 21-deoxy-11-oxo-steroid derivatives led to a significant decrease in the apparent K_m value (to about one-third to one-seventh) and a moderate decrease in the apparent V_{max} value (to about five-sixths to one-half). Introduction of a 17α-hydroxyl group into 21-hydroxy-steroid derivatives caused the apparent V_{max} value to increase by about 1.8- to 11-fold, but caused little or no decrease in the apparent K_m value. These results suggest that the hydroxyl group at the 21- or 17α-position directly restricted the conformation and the orientation of the 20-oxo group towards the catalytic site of the enzyme and influenced the hydrogen transfer stage in the catalytic process, and also that, of the substituents at the 21- and 17α-positions, the latter may preferentially affect the reaction efficiency of the 20-oxo group. The presence of an oxo group at C-11 had an indirect influence on the effects of the substituents at the 21- and 17α-positions.

The optimum orientation of the 20-oxo group for the catalytic reaction may occur in 21-deoxy-17-deoxy-11-deoxy-steroid derivatives. In an ideal ternary complex, the 20-oxo group of the steroid may project towards the β-face of the steroid ring and the conformation between the 20-oxo group and α-hydrogen of C-17 is nearly staggered, while that between C-21 and the α-hydrogen of C-17, looking along the C-17 to C-20 axis, is in a skew form; the 20-oxo group is orientated rather far from the methyl group at the β-position of C-13 and more towards the β-chain of C-16.

Keywords——steroid; 20-oxo-steroid; 20β-hydroxysteroid dehydrogenase; structural requirements; K_m and V_{max} for 20-oxo-steroids

In a series of studies on steroid-protein interactions, we have used 20β-hydroxysteroid dehydrogenase[2] [EC 1.1.1.53] from *Streptomyces hydrogenans* as a model protein and investigated its interactions with various pregnan-20-one derivatives in order to study some general aspects of steroid-protein interactions as well as to elucidate the basic mechanisms involved in the enzyme reaction. This enzyme transfers hydrogen from NADH to various 20-oxo-steroids to give the corresponding 20β-hydroxy derivatives.[3]

1) Location: *1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158, Japan.*
2) H.J. Hübener, F.G. Sahrholz, J. Schmidt-Thome, G. Nesemann, and R. Junk, *Biochim. Biophys. Acta*, **35**, 270 (1959).
3) H.J. Hübener and C.O. Lehmann, *Z. Physiol. Chem.*, **313**, 124 (1958); H.D. Henning and J. Zander, *Z. Physiol. Chem.*, **330**, 31 (1962).

The preceding work on the substrate specificity of this enzyme[4] has revealed that 20-oxo-steroids having a bulky substituent(s) at C-21 and/or C-17 did not serve as substrates, while steroids having a bulky group at C-3 or C-11 were utilized by the enzyme. The presence of a substituent at C-16 of 20-oxo-steroids resulted in almost complete loss of substrate reactivity. The configurational relationship between the plane of the steroid ring and 17β-side chain was important for the reactivity. It has also been shown that the substitution of a hydroxyl group for a hydrogen at C-21 and/or the introduction of a hydroxyl group at C-17 affects the reactivity of 20-oxo-steroids with the enzyme.[4] This suggested that the presence, nature, shape, and size of a substituent around the reacting 20-oxo group of steroids play a decisive role in the interaction with the catalytic site of the enzyme. The effect of a substituent at C-16 on the enzyme reaction was analyzed by kinetic experiments using 16-methyl derivatives of pregn-4-ene-3,20-dione as a substrate or inhibitor. It was deduced that the pyridine nucleotide coenzyme may be situated near C-16 of the steroid molecule (especially at the β-side) in the steroid-coenzyme-enzyme ternary complex in the catalytic process; the presence of a substituent at C-16 may inhibit the coenzyme interaction with the enzyme as well as with the reacting 20-oxo group.[4]

The present work was designed to elucidate more precisely the role of the regions around C-17 and C-21 on the interaction of the reacting 20-oxo group with the catalytic site of the enzyme.

Experimental

Materials——20β-Hydroxysteroid dehydrogenase [EC 1.1.1.53] from *Streptomyces hydrogenans* was obtained from Boehringer Mannheim GmbH, West Germany, and its purity was checked as described previously.[4] Most of the steroid used in this study were purchased from Sigma Chemical Co., U.S.A., E. Merck AG, West Germany, and Fluka AG, Switzerland. Pregn-4-ene-3,20-dione and 11β,17,21-trihydroxypregn-4-ene-3,20-dione were standard substances from the National Institute of Hygienic Sciences, Tokyo. NADH was purchased from Sigma Chemical Co., U.S.A. and Oriental Yeast Co., Tokyo.

Assay of 20β-Hydroxysteroid Dehydrogenase Activity——The enzyme activity was assayed at 25° by measuring the decrease in absorption of NADH at 340 nm under the conditions described in the previous paper,[4] except that the concentration of steroids was as indicated in the table and graphs. The enzyme was diluted with 5 mM sodium phosphate buffer (pH 7.0) containing 20% glycerol.

Concentrations of Enzyme, NADH, and Steroids——Concentrations of the enzyme, NADH, and steroids were determined by the methods described in our previous paper.[4]

Kinetic Measurements——The initial reaction rate was determined at 7—12 substrate concentrations covering as wide a range as possible, and each measurement was done at least twice. Linear regressions of the reciprocal of the initial reaction rate against the reciprocal of the substrate concentration were calculated by using the weighting procedure of Wilkinson.[5] The apparent V_{max} and apparent K_m values were the reciprocals of the intercepts of these regression lines with the ordinate and abscissa, respectively.[6]

Results and Discussion

Effect of a C-21 Hydroxyl Group on the Enzyme Reaction

Since 20-oxo-steroids having a bulky group at C-21 were ineffective or very poor substrates,[4] the C-21 position of a steroid may be able to interact only with a fairly specific region of the active site of the enzyme. On the other hand, 20-oxo-steroids having a hydroxyl group in place of the hydrogen atom at C-21 were utilized by the enzyme. However, the substitution generally produced an alteration in the reactivity of the parent compounds with the enzyme, and the extent of this effect appeared to depend further on the presence of a hydroxyl group at C-17, or an oxo or a hydroxyl group at C-11.[4] This may offer some clue as to the nature of the interaction of steroids with the catalytic site of the enzyme. Therefore, more precise kinetic

4) J. Kawamura, T. Hayakawa, and T. Tanimoto, *Chem. Pharm. Bull.*, **28**, 437 (1980).
5) G.N. Wilkinson, *Biochem. J.*, **80**, 324 (1961).
6) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

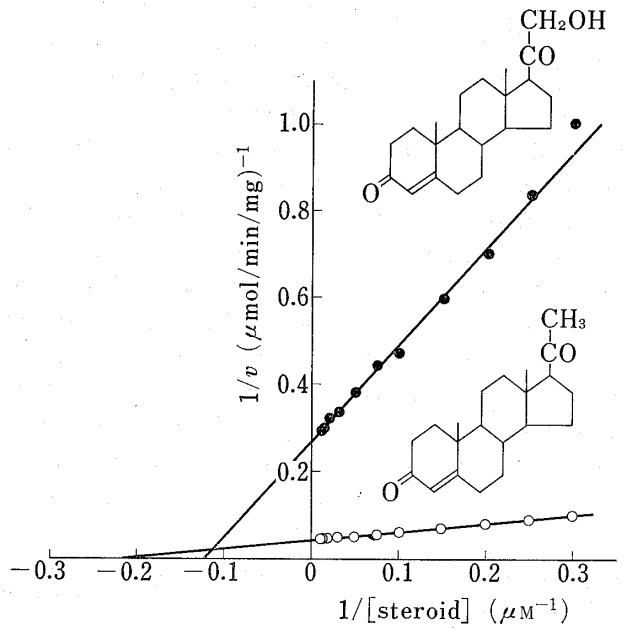

Fig. 1. Changes of K_m and V_{max} induced by the Introduction of a Hydroxyl Group at C-21 of a 17-Deoxy-steroid

—○—, pregn-4-ene-3,20-dione (IA-1).
—●—, 21-hydroxypregn-4-ene-3,20-dione (IB-1).

experiments were carried out, using various 21-hydroxy derivatives of 20-oxo-steroids, to obtain further information on the structural features required for steroid recognition by the enzyme. The initial velocities were measured with various 20-oxo-steroids as variable substrates and 150 μM NADH as a fixed substrate at pH 6.4 and 25°. The data were plotted by the method of Lineweaver and Burk,[6] and the kinetic parameters of the 21-deoxy derivatives were compared with those of corresponding 21-hydroxy derivatives.

i) 17-Deoxy-steroid Derivatives—In the case of 17-deoxy-11-deoxy-steroid derivatives, the characteristic effect of a hydroxyl group introduced at C-21 can be clearly seen by comparing pregn-4-ene-3,20-dione (IA-1) and 21-hydroxypregn-4-ene-3,20-dione (IB-1) (Fig. 1). Change of IA-1 to IB-1 resulted in a slight increase (about 1.8-fold) in the apparent K_m value

Table I. Kinetic Constants of Various 20-Oxo-steroids

Steroid (Compd. No.)	Concentration (μM)	Apparent K_m (μM)	Apparent V_{max} (μmol/min/mg)
a) 17-Deoxy-21-deoxy-steroids			
Pregn-4-ene-3,20-dione (IA-1)	3.3—100	4.5	23.5
Pregn-4-ene-3,11,20-trione (IA-3)	80 —400	148	96.7
11β-Hydroxypregn-4-ene-3,20-dione (IA-8)	50 —300	505	10.4
3α-Hydroxy-5β-pregnane-11,20-dione (IIIA-6)	26.7—200	604	40.0
3β-Hydroxypregn-5-en-20-one (IVA-1)	3.3— 33.3	2.9	7.6
3β-Hydroxy-6-methylpregn-5-en-20-one (IVA-2)	3.3— 50	1.8	6.2
b) 17-Deoxy-21-hydroxy-steroids			
21-Hydroxypregn-4-ene-3,20-dione (IB-1)	4 —100	8.1	3.7
21-Hydroxypregn-4-ene-3,11,20-trione (IB-3)	50 —300	253	12.0
11β,21-Dihydroxypregn-4-ene-3,20-dione (IB-6)	100 —400	576	1.2
3α,11β,21-Trihydroxy-5β-pregnan-20-one (IIIB-3)	66.7—200	353	0.2
c) 17-Hydroxy-21-deoxy-steroids			
17-Hydroxypregn-4-ene-3,20-dione (IA-2)	3.3—100	4.2	9.9
17-Hydroxypregn-4-ene-3,11,20-trione (IA-4)	20 —200	52.9	52.2
3α,17-Dihydroxy-5β-pregnan-20-one (IIIA-5)	4 — 66.7	1.8	3.6
3α,17-Dihydroxy-5β-pregnane-11,20-dione (IIIA-7)	33.3—200	84.0	32.8
3β,17-Dihydroxypregn-5-en-20-one (IVA-3)	3.3— 33.3	2.2	5.7
3β,17-Dihydroxy-6-methylpregn-5-en-20-one (IVA-4)	3.3— 50	2.0	4.6
d) 17-Hydroxy-21-hydroxy-steroids			
17,21-Dihydroxypregn-4-ene-3,20-dione (IB-2)	3.3—100	5.5	9.8
17,21-Dihydroxypregn-4-ene-3,11,20-trione (IB-4)	33.3—200	83.5	21.4
11β,17,21-Trihydroxypregn-4-ene-3,20-dione (IB-7)	100 —400	317	3.4
3α,17,21-Trihydroxy-5β-pregnan-20-one (IIIB-1)	3.3— 66.7	3.3	4.4
3α,17,21-Trihydroxy-5β-pregnane-11,20-dione (IIIB-2)	50 —300	121	8.0
3α,11β,17,21-Tetrahydroxy-5β-pregnan-20-one (IIIB-4)	66.7—400	390	2.2

and a large decrease (to about one-sixth) in the apparent V_{max} value. Similar changes in kinetic constants were also found between pregn-4-ene-3,11,20-trione (IA-3) and its 21-hydroxy derivative (IB-3) and between 11β-hydroxypregn-4-ene-3,20-dione (IA-8) and its 21-hydroxy derivative (IB-6) (Table I-a and -b). The apparent K_m values of the 17-deoxy-11-oxo (IA-3) and 17-deoxy-11-hydroxy (IA-8) derivatives were increased 1.7- and 1.1-fold by the introduction of a hydroxyl group at C-21, and the apparent V_{max} values decreased to one-eighth and one-ninth, respectively.

Since the introduction of a hydroxyl group into C-21 resulted in a significant decrease in the apparent V_{max} value with a rather smaller increase in the apparent K_m value, as described above, it was considered that the substitution at C-21 may markedly affect the step of hydrogen transfer between the 20-oxo group of steroids and NADH in the ternary complex rather than the steroid binding process itself. If a hydroxyl group is substituted for a hydrogen atom at C-21, it is possible that the spatial position of the 20-oxo group may be restricted unfavorably as a result of steric interference with this primary alcohol group and, consequently, the efficiency of hydrogen transfer would be reduced. It seems likely that, in the case of 21-deoxy derivatives, C-20 may have relatively free rotation for efficient interaction between the catalytic site of the enzyme and the 20-oxo group, since the C-C bond of the side chain attached to C-17 is a σ bond and the three hydrogen atoms of the methyl group (C-21) are equivalent.

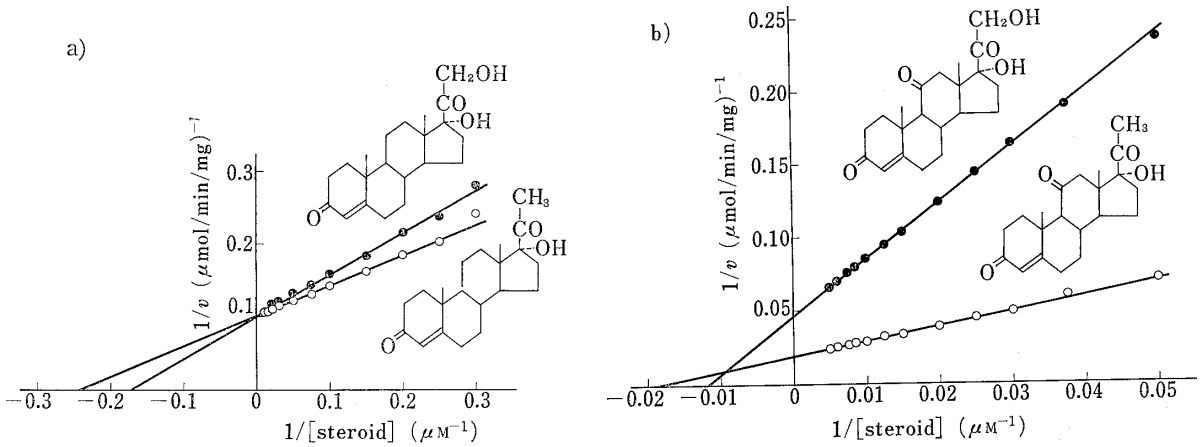

Fig. 2. Changes of K_m and V_{max} induced by the Introduction of a Hydroxyl Group at C-21 of 17-Hydroxy-steroids

a) —○—, 17-hydroxypregn-4-ene-3,20-dione (IA-2);
—●—, 17,21-dihydroxypregn-4-ene-3,20-dione (IB-2).
b) —○—, 17-hydroxypregn-4-ene-3,11,20-trione (IA-4);
—●—, 17,21-dihydroxypregn-4-ene-3,11,20-trione (IB-4).

ii) 17-Hydroxy-steroid Derivatives——The characteristic effect of the substitution of a hydroxyl group of C-21 in 17-hydroxy-11-deoxy derivatives can be seen by comparing the results for 17-hydroxypregn-4-ene-3,20-dione (IA-2) and 17,21-dihydroxypregn-4-ene-3,20-dione (IB-2) (Fig. 2-a). The substitution caused little decrease in the apparent V_{max} value, in contrast to the case of 17-deoxy derivatives, though the increase in the apparent K_m (about 1.3-fold) was the same. A similar tendency was also observed in comparing 3α,17-dihydroxy-5β-pregnan-20-one (IIIA-5) with 3α,17,21-trihydroxy-5β-pregnan-20-one (IIIB-1) (Table I-c and -d). On the other hand, the presence of the 11-oxo group alters the situation somewhat, as shown in Fig. 2-b. Changes of substrate from 17-hydroxypregn-4-ene-3,11,20-trione (IA-4) and 3α,17-dihydroxy-5β-pregnane-11,20-dione (IIIA-7) to 17,21-dihydroxypregn-4-ene-3,11,-20-trione (IB-4) and 3α,17,21-trihydroxy-5β-pregnane-11,20-dione (IIIB-2), respectively, produced a decrease in the apparent V_{max} value (Table I-c and -d) in contrast to the case of 17-hydroxy-11-deoxy-steroid derivatives. However, the extent of the decrease (to about one-half to one-fourth) was small compared with that in 17-deoxy-11-oxo derivatives (to about one-

eighth). On the introduction of a hydroxyl group at C-21 of 17-hydroxy-11-oxo derivatives, the apparent K_m values increased slightly (1.4- to 1.6-fold) as in the case of 17-deoxy-steroid derivatives. This suggests that the conformation of the 20-oxo group may be strongly restricted by the 17α-hydroxyl group attached to the steroid ring, and hence it was not greatly affected by replacement of the hydrogen of C-21 with a hydroxyl group. Some indirect effect of an oxo group at C-11 was also suggested.

Effect of Introduction of a 17α-Hydroxyl Group on the Kinetic Constants

Figure 3 shows an example of the effect of introduction of a 17α-hydroxyl group on the kinetic constants of 21-deoxy-11-deoxy derivatives (IVA-2, IVA-4). The introduction of a 17α-hydroxyl group caused little change in the apparent K_m value and a slight decrease in the apparent V_{max} value. Similar results were obtained when the kinetic constants of IA-1 and 3β-hydroxypregn-5-en-20-one (IVA-1) were compared with those of IA-2 and 3β,17-dihydroxypregn-5-en-20-one (IVA-3), respectively (Table I-a and -c). This suggests that a 17α-hydroxyl substituent may restrict the free rotation of the 20-oxo group and thereby shift the 20-oxo group slightly from the most advantageous orientation for the enzymic reaction.

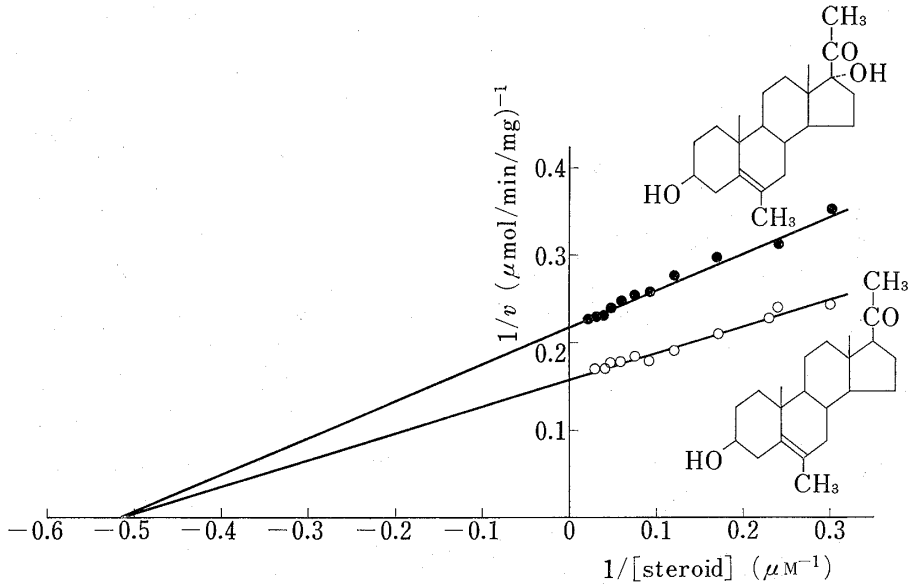

Fig. 3. Changes of K_m and V_{max} induced by the Introduction of a Hydroxyl Group at C-17 of a 21-Deoxy-11-deoxy-steroid

—○—, 3β-hydroxy-6-methylpregn-5-en-20-one (IVA-2);
—●—, 3β-17-dihydroxy-6-methylpregn-5-en-20-one (IVA-4).

The effect of introduction of a 17α-hydroxyl group in 21-deoxy-11-oxo derivatives can be seen by comparing IA-3 and 3α-hydroxy-5β-pregnane-11,20-dione (IIIA-6) with their 17α-hydroxy derivatives, IA-4 and IIIA-7 (Fig. 4, Table I-a and -c). A significant decrease (to about one-third and one-seventh, respectively) in the apparent K_m value and a slight decrease (to about one-half and five-sixths, respectively) in the apparent V_{max} value were common features in these cases. It seems probable that a 17α-hydroxyl group may partially restore the reduced affinity of steroids for the enzyme caused by the presence of the 11-oxo group.

An example of the effect of a 17α-hydroxyl substituent in 21-hydroxy derivatives is shown in Fig. 5. It should be noted that the apparent V_{max} value was increased by the introduction of a 17α-hydroxyl group into 21-hydroxy derivatives, although its introduction into 21-deoxy derivatives induced a decrease in the apparent V_{max} value. Other examples (IB-1 and IB-2, IB-3 and IB-4, IIIB-3 and IIIB-4) also showed a similar tendency (Table I-b and -d). The increase in the apparent V_{max} value was found to be about 1.8- to 11-fold. On the other hand,

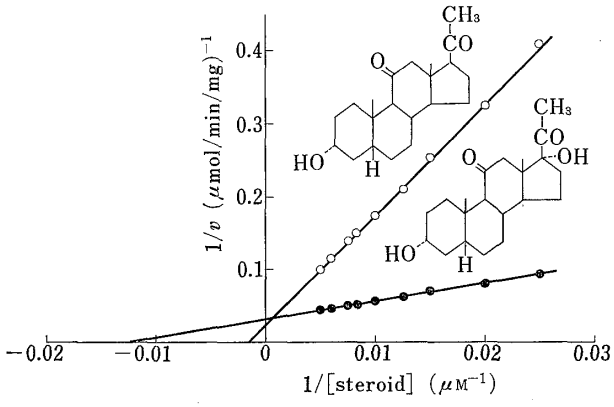

Fig. 4. Changes of K_m and V_{max} induced by the Introduction of a Hydroxyl Group at C-17 of a 21-Deoxy-11-oxo-steroid

—○—, 3α-hydroxy-5β-pregnane-11,20-dione (IIIA-6);
—●—, 3α,17-dihydroxy-5β-pregnane-11,20-dione (IIIA-7).

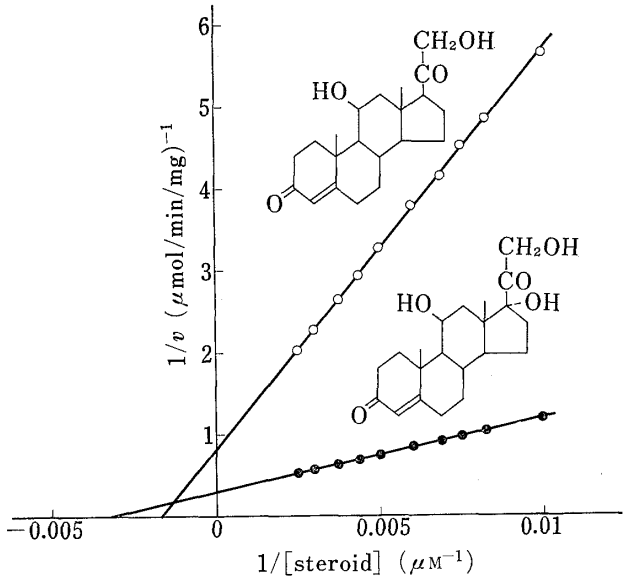

Fig. 5. Changes of K_m and V_{max} induced by the Introduction of a Hydroxyl Group at C-17 of a 21-Hydroxy-steroid

—○—, 11β,21-dihydroxypregn-4-ene-3,20-dione (IB-6);
—●—, 11β,17,21-trihydroxypregn-4-ene-3,20-dione (IB-7).

little or no decrease in the apparent K_m value was caused by a 17α-hydroxyl substituent (to between one-third and unchanged). To explain the enhancing effect of the 17α-hydroxyl group on the apparent V_{max} values of 21-hydroxy derivatives, conformational changes of the reacting 20-oxo group induced by the 21-hydroxyl group and/or 17α-hydroxyl group may again have to be taken into consideration. In 17α,21-dihydroxy derivatives, both hydroxyl groups can affect the conformation of the 20-oxo group and it is likely that the orientation of the 20-oxo group for hydrogen transfer may be improved by the presence of the 17α-hydroxyl group. In other words, the significant strain on the 20-oxo group due to the presence of the 21-hydroxyl group may be partially released by the 17α-hydroxyl substituent. It is of interest that the enhancing effect of the 17α-hydroxyl group on the V_{max} of 21-hydroxy derivatives occurred regardless of the presence or absence of an oxo or a hydroxyl group at C-11. Since all 17-deoxysteroids having an oxo or a hydroxyl group at C-11 have high K_m values compared with those of 11-deoxy derivatives, it is assumed that an oxo or a hydroxyl group at C-11 may tend to separate the steroid ring from the enzyme, possibly leading to some restriction of orientation or distance from the active site of the enzyme. Nevertheless, the findings that 11-deoxy derivatives as well as 11-oxo and -hydroxy derivatives could produce similar enhancement of the hydrogen transfer process (increase in V_{max}) upon the introduction of a 17α-hydroxyl group strongly suggest that the role of the 17α-hydroxyl group may at least partly involve the restoration of a more favorable orientation of the 20-oxo group rather than simply a recovery of affinity for the enzyme.

It seems likely that, in the enzyme-coenzyme-steroid ternary complex, the 20-oxo group of steroids normally projects towards the β-face of the molecule. If the 20-oxo group projected toward the α-face of the steroid ring, severe steric hindrance could occur between the 13β-methyl group (C-18) and C-21, and furthermore, the introduction of a hydroxyl group into the C-17 α-position would bring about a greater inhibitory effect as a result of an eclipsed interaction of the 17α-hydroxyl group with the 20-oxo group. In addition, a considerable decrease in the reaction rate upon the introduction of a C-16/C-17 double bond[4] cannot be explained unless one assumes that the 20-oxo group projects towards the β-face of the steroid skeleton. If it was situated in the α-face, the configuration of the side chain at C-17 resulting from the intro-

duction of a C-16/C-17 double bond, in which the C-17/C-20 bond is almost planar with respect to the steroid ring, would cause the 20-oxo group to interact more closely with the active site of the enzyme and would enhance the efficiency of hydrogen transfer.

It is also likely that fairly strict specificity of conformation and orientation of the 20-oxo group are required for effective enzymic action. In the ternary complex, the ideal spatial position of the 20-oxo group at the moment of hydrogen transfer should be that giving the most efficient interaction with the hydrogen donor at the active site of the enzyme. The finding that the highest reactivity was found in 21-deoxy-17-deoxy derivatives suggests that the most advantageous conformation and orientation of the 20-oxo group occurs when the conformation between the 20-oxo group and α-hydrogen of C-17 is nearly staggered and that between C-21 and the α-hydrogen of C-17, looking along the C-17 to C-20 axis, is in a skew form with the 20-oxo group positioned rather far from the 13β-methyl group (C-18). Such relationships can certainly be attained with relatively free rotation of the C-17/C-20/C-21 bonds and equivalency of the three hydrogens at C-21. The presence of a substituent at C-21 and/or C-17α may lead to subtle conformational and orientational changes of the 20-oxo group and thereby significantly affect the efficiency of the hydrogen transfer process.

It also appeared that the presence of an oxygen atom at C-11 indirectly influenced the effect of substituents at C-21 and C-17 on the orientation of the 20-oxo group with respect to the catalytic site through a repulsive effect on the binding site of the enzyme. The role of the oxygen atom at C-11 will be discussed in detail in a subsequent paper.

In conclusion, the conformational relationships among substituents at C-17α, 20, and 21 clearly played an important role in the interaction between 20-oxo-steroids and 20β-hydroxysteroid dehydrogenase, as described in this paper, and this may have implications for the interactions between steroid hormones or drugs and proteins such as enzymes, steroid receptors, and steroid-binding proteins.