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Studies on Δ5→4-3-Oxo Steroid Isomerases. I. An Extraction Model for Enzymatic Activity*

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ABSTRACT: *Pseudomonas testosteroni* Δ5→4-3-oxo steroid isomerase has been tested for analyzing the enzyme–substrate or inhibitor complex formation in terms of an extraction process of steroid from the aqueous medium by the hydrophobic part of the protein. 5-Androstene-3,17-dione, 5-estrene-3,17-dione, and 5-pregnene-3,20-dione have been used as substrates, and 19-nortestosterone, 4-estrene-3,17-dione, progesterone, and 19-norprogesterone as competitive inhibitors, varying the methanol concentration of the reaction medium. Log $1/K_m$ and log $1/K_i$, according to the case, plotted *vs.* methanol concentration, decrease linearly. Log K_p (partition coefficient of the steroids between isooctane and various methanol–water mixtures) *vs.* methanol concentration decreases also linearly. These results are in favor of the extraction model.

Parallel straight lines are obtained with a given steroid for $1/K_m$ or log $1/K_i$ *vs.* log K_p, which indicate the participation of the hydrophobic groups of steroids to the enzyme–substrate binding. The V_{max} increase induced by methanol for 5-estrenedione and 5-pregnenedione isomerization (but not for 5-androstenedione) is discussed in terms of relative affinity of substrate and reaction product in each case.

Various 19-nor-Δ4-3-oxo steroids have been used at a given methanol concentration. Among the structural features of the steroid molecule which interfere in steroid–protein interaction, the substitution of the C_{19} methyl group by hydrogen could increase the affinity by favoring enol formation.

The Δ5→4-3-oxo steroid isomerase of *Pseudomonas testosteroni* (EC 5.3.3.1.)[1] provides a rather simple model of direct interaction between a steroid molecule and a protein. This enzyme, studied by Talalay, requires no prosthetic group and the isomerization reaction occurs by intramolecular hydrogen transfer without incorporation of protons from the medium (Kawahara *et al.*, 1962; Wang *et al.*, 1963).

Steroid substrates are rigid hydrocarbon molecules of low solubility and the formation of enzyme–substrate complex may largely involve hydrophobic interactions. As changes in the structure of water surrounding the nonpolar groups play a major role in these interactions, the nature of the solvent medium appears to be of particular importance.

Changes in composition of the reaction medium and their effects on enzymatic reactions have been extensively studied and give some information on the nature of enzyme–substrate interactions. A number of water-

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[1] Isomerase will be used in this paper.

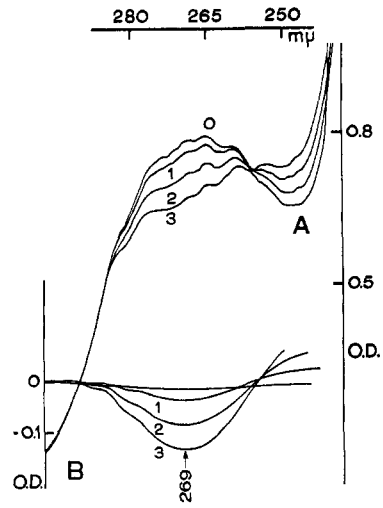


FIGURE 1: Ultraviolet spectrophotometry of isomerase. (A) Ultraviolet spectrum. (0) In medium 0.02 M Tris-HCl (pH 7.5) without methanol, or after incubation 48 M at 4° with up to 50% methanol and dialysis. (1) After 48-hr incubation at 6° in Tris-HCl and 6.6% methanol; (2) same as 1 but with 13.3% methanol; (3) same as 1 but with 20.0% methanol. (B) Difference spectrum.

miscible organic solvents have been found to act as inhibitors, the major effect being a decrease in affinity for the substrate as the concentration of solvent is raised.

Several models have been proposed and are based mainly on the effects of solvents on the medium dielectric constant and consequently on the electrostatic interaction between enzyme and substrate, on the binding of the solvent molecules to the specific sites of the protein, or on both (Applewhite *et al.*, 1958; Miles *et al.*, 1962; Clement and Bender, 1963). Applewhite *et al.* (1958) have also suggested a partition of substrate of low solubility between two phases, the hydrated enzyme and the water–solvent system. For the isomerase, it has been proposed to compare the enzyme–substrate complex formation to an extraction process, from the aqueous solvent, of the hydrocarbon steroid molecule by the hydrophobic part of the protein (Alfsen *et al.*, 1966); if this were true, the partition model might also apply to the effects produced by addition of organic solvent in the reaction medium.

The extraction model has been developed on a theoretical basis. It was proposed that substrate or inhibitor was extracted from the aqueous medium by an hydrocarbon area on the active site of the enzyme (Miles *et al.*, 1963; Hymes *et al.*, 1965; Wildnauer and Canady, 1966).

An investigation of the kinetic constants of the isomerase activity (turnover constant and apparent affinity constant for several substrates and competitive inhibitors) in varying concentrations of methanol is reported in this paper. Correlation between substrate polarity and affinity was studied by comparing the affinity constant for a given steroid and its partition coefficient between aqueous and hydrocarbon (isooctane) phases. There are actually also variations in polarity obtainable from the very structure of steroids and various compounds have been compared.

However, the formation of the enzyme–substrate complex cannot be interpreted only in terms of hydrophobic forces concerned with this extraction model, since the steroid molecules have also hydrophilic groups, such as ketones or hydroxyl groups. These groups modify likely the water structure in the steroid neighborhood and hence hydrophobic interactions between enzyme and substrate; they may also be responsible for more specific interactions with hydrophilic groups of the enzyme, and orientation of the substrate by group dipole moment toward the active site of the enzyme. The fact, suggested by Wang *et al.* (1963), that enolization of the steroid might occur in the formation of the isomerase–inhibitor complex, is relevant to these considerations. Therefore, some factors that control the formation and the stability of enolate anions of the Δ4-3-oxo steroids, already pointed out by Ringold (Subrahmanyam *et al.*, 1966), will be considered in their relation with isomerase activity.

Experimental Procedure

Isomerase Preparation. Isomerase is purified according to the procedure of Talalay and Boyer (1965), from *P. testosteroni* (Strain ATCC 11.996) grown on progesterone and lyophilized.

The enzyme used in these studies corresponds to a fraction obtained by chromatography on DEAE-cellulose, precipitated by $(NH_4)_2SO_4$ between 0 and 70%, and further dialyzed. The specific activity of the final preparation = 40,000 units/mg. The ultraviolet spectrum of the preparation exhibits the hyperfine structure reported for the pure crystalline enzyme and the extinction coefficient at 280 mμ is identical (Kawahara *et al.*, 1962). Electrophoresis on polyacrylamide gel plate has shown one major band from which the enzymatic activity could be recovered by elution.

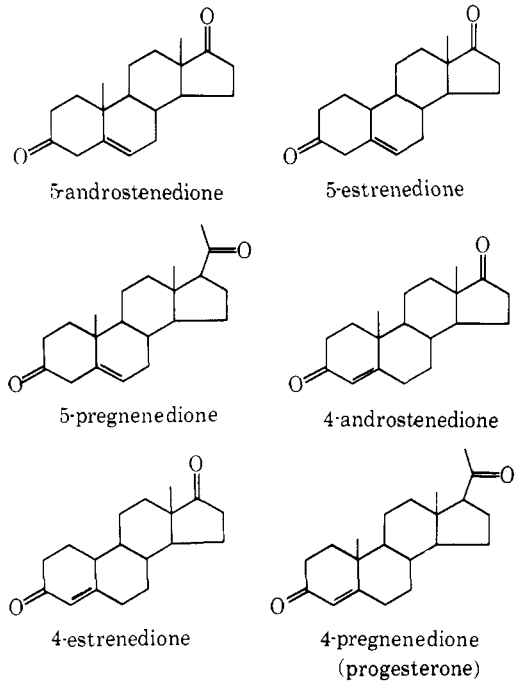
A spectrophotometric study has been done (Figure 1), changing the methanol concentration of the medium. Reversibility of the ultraviolet spectrum is obtained after dialysis out of any concentration of methanol up to 50%. The activity is also unchanged after incubation in methanolic medium up to 48 hr at 4°.

Kinetics. Enzyme dilution is made in 0.2% crystalline bovine serum albumin. The reaction mixture contains 0.02 M Tris-HCl (pH 7.5), different amounts of methanol, substrates, and inhibitors in a final volume of 3.0 ml. Methanol has been added in place of an equivalent volume of buffer and additions of solvent assumed to result in strictly additive volume changes; percentages are volumes/100 volumes. The slight change in ionic strength has been shown to be without effect on the reaction rate. The methanol concentrations (1.7–20%) have been shown to be without effect on the pH of Tris buffers (Wheland, 1960) and the reaction rate is essentially the same between pH 6 and 9.

Changes in absorbancy at 248 mμ have been followed with a Beckman DB recording spectrophotometer fitted with a thermostated cell compartment. Unless otherwise stated all assays are performed at 21° and, under those conditions, spontaneous isomerization is negligible. Initial rates were recorded in all cases.

K_m and V_{max} are obtained from the intercepts of the

reciprocal plots of the kinetic data. V_{max} are extrapolated values since, in most cases, saturation levels could not be obtained because of low substrate solubility. Isomerase concentration is inferred from the observed activity under standard assay conditions using the data of Talalay. Three substrates are used: 5-androstenedione, 5-estrenedione, and 5-pregnenedione.



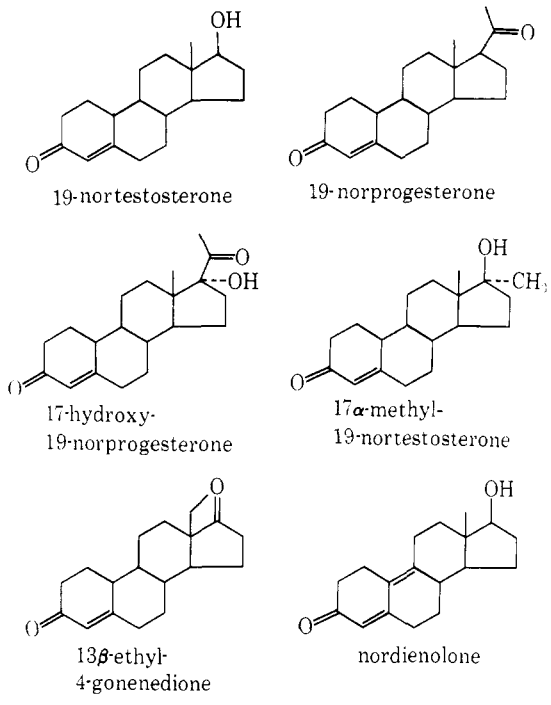
Various Δ4-3-oxo steroids have been tested as inhibitors of the reaction: 4-androstenedione, 4-estrenedione, and 4-pregnenedione which correspond to the three substrates; 19-nortestosterone, 19-norprogesterone, 17-hydroxy-19-norprogesterone, 17α-methyl-19-nortestosterone, 13β-ethyl-4-gonenedione, and nordienolone, which have in common the absence of a 19-methyl group.

Inhibition, when observed, is strictly competitive. K_i's are obtained from the point of intersection of the lines $1/v$ *vs.* i obtained at three substrates concentrations (v being the initial velocity and i the inhibitor concentration).

Extraction Coefficients. Δ4-3-Oxo steroid partitions between water–methanol and isooctane phases have been studied. The extraction coefficient is calculated as the ratio of the concentration in isooctane to the concentration in water–methanol at equilibrium.

The extraction coefficient cannot be measured for the Δ5-3-oxo steroids since partial isomerization occurs during the time necessary to reach equilibrium. It will be assumed in the following discussion that the values obtained for the Δ4-3-oxo steroids can be applied to the corresponding Δ5 derivatives.

Steroids are dissolved in the aqueous phase, *i.e.*, Tris-HCl (0.02 M, pH 7.5) containing varying amounts of methanol as for the kinetic measurements. Enough material is utilized to have a starting optical density at 248 mμ of about 0.6 in cells of 1-cm light path. An aliquot is transferred to a cylindrical funnel along with a



suitable volume of isooctane. The ratio of the volume of water phase to that of the hydrocarbon phase varies from 30 to 2, depending upon the polarity of the steroid. The total volume is between 25 and 30 ml. The vials are agitated by rotation at 21° for 8 hr, equilibrium being reached in 2–4 hr. After agitation the flasks stand for a few minutes and an aliquot of the aqueous phase is removed.

The solute concentrations in both phases are spectrophotometrically determined in the aqueous phase before and after equilibrium has been reached. It is read at 248 mμ (310 mμ for nordienolone and 13β-ethyl-4-gonenedione) in a Beckman DB spectrophotometer. The concentration of solute in the hydrocarbon phase is estimated by difference.

In the following discussion, the methanol concentrations indicated correspond to initial values and the distribution of methanol between water and isooctane is considered as negligible.

Results

Effect of Methanol on the Rate of Enzymatic Isomerization. As shown in Figure 2 the rate of isomerization is modified by the addition of methanol but the resulting effect depends upon the substrate. Raising the concentration of methanol decreases the rate of isomerization of 5-androstenedione, but a stimulation is first obtained with 5-estrenedione and 5-pregnenedione as substrates, up to a maximum beyond which a decrease is observed. Dioxane exerts a similar effect.

As steroids are compounds of low solubility, it must be pointed out that concentration in the assay mixture is always below the saturation for all substrates. Furthermore, 5-pregnenedione is less polar than 5-androstenedione, itself less polar than 5-estrenedione, ruling out an explanation based on the solubilization effect.

TABLE I: Influence of Methanol on the Enzymatic Isomerization of Various Δ5-3-Oxo Steroids.[a]

% Methanol	1.7	3.3	7	8.5	10	13	17	20
5-Androstenedione								
$K_m \times 10^4$ M		2.9		6.0		9.1		
V_{max}		10		9		5.6		
5-Estrenedione								
$K_m \times 10^4$ M	1.0	1.3	2.1			7.7		
V_{max}	3.3	3.8	4.8			4.7		
5-Pregnenedione								
$K_m \times 10^4$ M					0.5	0.9	2.7	4.5
V_{max}					3.9	6.6	13	15

[a] K_m (10^{-4} M) and V_{max} (moles of substrate transformed per minute per micromole of enzyme) are determined in 0.02 Tris-HCl buffer (pH 7.5) at 21° and at the indicated concentrations (v/v) of methanol.

Effect of Methanol on the Kinetics of the Reaction and on Competitive Inhibition. As shown in Table I, K_m for the three substrates increases with increasing methanol concentration. Plot of log $1/K_m$ *vs.* methanol concentration is linear (Figure 3). The more complex effects on V_{max} appear to be dependent upon substrate: while decreasing for 5-androstenedione, V_{max} increases for 5-estrenedione and for 5-pregnenedione, raising methanol concentration from 10 to 20%, resulted in a fourfold increase.

A number of Δ4-3-oxo steroids act as competitive inhibitors. For some of them, K_i measured at different methanol concentrations is given in Table II. A plot of log $1/K_i$ *vs.* methanol concentration is linear. Although it is uncertain whether or not K_m represents a true association constant, these results can be taken as an indication that methanol may act in the formation of the complex between enzyme and substrate or inhibitor. Among inhibitors, it should be noted that 4-estrenedione and 4-pregnenedione are isomerization products of two of the substrates, precisely those for which enhancement of rate by methanol was observed.

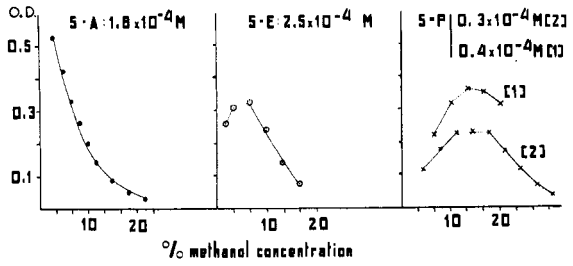


FIGURE 2: Effect of methanol on the rate of isomerization of Δ5-3-oxo steroids by isomerase. Cuvets contain 0.02 M Tris-HCl (pH 7.5) and methanol (v/v) in a final volume of 3.0 ml. Pure methanol is added to the cuvets in place of an equivalent volume of buffer. The steroids (5-P, 5-pregnenedione; 5-E, 5-estrenedione; 5-A, 5-androstenedione) are added as methanolic solutions to give the indicated final concentrations. Reactions are initiated by addition of 25 μl of enzyme and rates of formation of the Δ4-3-oxo product are followed at 248 mμ at 21°.

Correlation between Affinity Constants and Partition Coefficient. POLARITY CHANGE BY VARYING METHANOL CONCENTRATION. In Table III is reported the partition coefficient, K_p, of various steroids between an aqueous phase and an organic solvent, which is function of the amount of methanol in the water phase. A plot of log K_p *vs.* methanol concentration is linear. Therefore methanol appears to affect both the extraction process and the formation of the steroid–enzyme complex in the same direction and following an exponential law.

Straight lines are also obtained when plotting, for a given steroid, log $1/K_m$ or log $1/K_i$ (log K') *vs.* log K_p (Figure 4). All the lines obtained for the various substrates and inhibitors are strikingly parallel with an average slope of 3.0, individual values ranging from 2.8 to 3.3.

POLARITY CHANGE BY STRUCTURAL VARIATIONS OF STEROIDAL INHIBITORS. In Table IV are given K_i and K_p for various Δ4-3-oxo steroids inhibitors of the reaction, measured at the same methanol concentration. All these steroids are 19-nor derivatives, actually more potent inhibitors than corresponding 19-methyl compounds. Rings A and B, where the reaction occurs, are the same, and structural changes are rather limited.

It can be seen that K_i's for 17-hydroxy and 17-keto steroids have the same magnitude, between 10 and 27 $\times$ 10^{-6} M; the partition coefficients are also in the same range; the presence of a side chain in 19-norprogester-

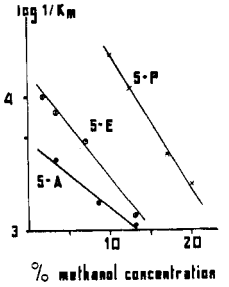


FIGURE 3: $1/K_m$ of isomerase as a function of methanol concentration for various substrates.

TABLE II: Influence of Methanol on Competitive Inhibition of Isomerase: Values of K_i.[a]

% Methanol	3.3	7	8.5	10	13	17
19-Nortestosterone	13	22		37		
4-Estrenedione	27		60		120	
Progesterone	6.4		18		100	
19-Norprogesterone	0.5	2.5		6.0		24

[a] K_i Values (10^{-6} M) for the mentioned Δ4-3-oxo steroids are determined at various concentrations of methanol. The substrate is 5-androstenedione. K_i is obtained from the point of intersection of the lines $1/v$ *vs.* i, obtained at 3 concentrations of substrate: 0.6×10^{-4}, 1.3×10^{-4}, and 2.5×10^{-4} M. The experimental conditions are the same as in Figure 1.

TABLE III: Partition Coefficient of Δ4-3-Oxo Steroids Inhibitors and/or Products of the Isomerase Reaction between a Water–Methanol Phase and an Isooctane Phase.[a]

% Methanol	1.7	3.3	7	8.5	10	13	17	20
4-Androstenedione		7.0		5.8		4.6		
19-Nor-4-androstenedione	4.2	3.6	3.2			2.0		
Progesterone		158		103	82	66	48	34
19-Norprogesterone		63	45		35		20	

[a] Steroids are dissolved in 0.02 M Tris-HCl (pH 7.5) containing methanol (v/v). The water–methanol phase transferred, along with isooctane into cylindrical funnel which is agitated by rotation at 21° for 8 hr. The partition coefficient, K_p, was given by the relation $K_p = [(OD_i - OD_{eq})/OD_{eq}]$[volume of the water–methanol phase/volume of the isooctane phase], OD_i and OD_{eq} being the initial and final optical density at 248 mμ of the water–methanol phase, OD_i is always around 0.5, and the ratio of water–methanol phase varied from 30 to 2, according to the steroid considered in order to have an OD_{eq} not less than 0.1. Total volume varies between 25 and 30 ml.

one results in a marked increase of both partition coefficient and affinity for the enzyme. This effect is also noted when comparing the affinity of the enzyme for the substrates 5-androstenedione and 5-pregnenedione (Table I). However the correlation between steroid polarity and affinity to the enzyme is far to be systematic. For instance 17-hydroxy-19-norprogesterone falls close to 19-norprogesterone in terms of affinity but is relatively very polar.

Enzyme Inhibitor Complex Using Δ4-3-Oxo Steroids with and without the 19-Methyl Group. Nineteen nor-Δ4,3-oxo steroid derivatives are potent inhibitors of the isomerization reaction as already observable from the data of Wang *et al.* (1963), using 19-nortestosterone, estradiol, and dihydroequilenin, the two latter phenol steroids being considered as enol form of 3-oxo-Δ1,4-diene compounds.

It can be seen in Table II, when comparing progesterone and 19-norprogesterone, that the absence of the C_{19} methyl group results in increased inhibition, this effect being independent of the methanol concentration. Likewise, whereas nortestosterone and norandrostenedione inhibited the enzyme, testosterone and androstene dione did not.

Discussion

Results indicate a close relationship between partition coefficient, K_p, derived from experiments using a *given steroid molecule* and *changing the aqueous phase polarity* and the constants (K_m and K_i) of affinity of substrates or inhibitors obtained from enzyme kinetics study.

This work deals more with affinity problems than with the mechanism of energy transfer from the enzyme to the substrate. The parameters defining the affinity of the substrate or inhibitor for the protein are approached by K_m or K_i values. They are related to factors controlling the accessibility to the reaction site, essentially hydrophobic interactions between substrate and the hydrophobic part of the protein, and dipole–dipole interactions leading to preferential orientation of substrate toward the active site.

The approach which uses varying methanol concentration in the aqueous medium has to be discussed. K_m and K_i values increase along with methanol concentration and, at least for inhibition, this could mean a decrease of affinity of the considered steroid for the enzyme when its solubility in the aqueous medium increases.

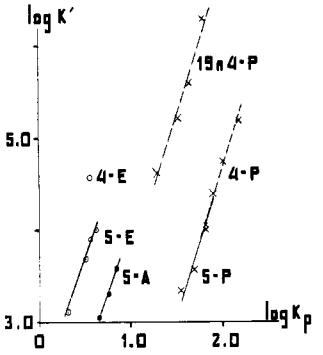


FIGURE 4: Plot of $1/K_m$ or $1/K_i$ (expressed as log K') of isomerase *vs.* log K_p. K_p, partition coefficient between isooctane and methanol–water phase. 4-P, progesterone (4-pregnenedione); 19n-4-P, 19-norprogesterone; 4-E, 4-estrenedione.

Actually the effect could be due either to a direct effect of methanol on the isomerase (as for ribonuclease, see Schrier *et al.*, 1965), or to some changes of the medium (for example, decrease of the dielectric constant), or to a modification of the solubilization state of the steroid molecule and therefore of its hydrophobic interactions with the enzyme. This last possibility seems more in agreement with the correlation between the observed changes of partition coefficient and the apparent affinity constant of the enzyme for the steroids, supporting the Canady model (Hymes *et al.*, 1965).

The fact that the slope of the linear function log $1/K_m$ or log $1/K_i$ *vs.* log $1/K_p$ is different from 1 (actually $\sim$3.0) indicates that the free-energy variations implicated in the affinity of the enzyme for substrates is proportional by a factor of $\sim$3 to the free energy dealing with the partition coefficient. This could indicate that in addition to the extraction process of the steroid by the hydrophobic part of the enzyme site, there is another energetic factor related to the hydrophilic group(s) of the substrate.

Methanol effect on V_{max} seems in contradiction to increase of K_m or K_i. However, the V_{max} increase occurs only for 5-estrenedione and 5-pregnenedione and the products, 4-estrenedione and progesterone, are inhibitors of the enzymatic isomerization (whereas 4-androstenedione does not inhibit 5-androstenedione isomerization under the experimental conditions). It is therefore possible that there is a V_{max} increase because there is a more important decrease of the inhibitor affinity than that of substrate. The role of substrate solubility should be ruled out since 5-androstenedione has a polarity intermediate to 5-estrenedione and 5-pregnenedione, and shows no V_{max} increase with increasing methanol. It has been observed that when there is undissolved substrate in the reaction medium, it acts as an inhibitor (Alfsen *et al.*, 1966). Since there is no saturation of isomerase by substrates which are of low solubility, it is difficult to see why there is no more formation of the enzyme–substrate complex when there is removal of the substrate from the medium which should displace the dissolved–undissolved substrate equilibrium. It leads to postulate that substrate excess can be inhibitor, or a certain form of solvated substrate molecule, since methanol addition increases the solubility of all substrates but does not always lead to a V_{max} increase.

TABLE IV: Comparison between K_i and K_p for Different 19-Nor-Δ4-3-oxo Steroids, Inhibitors of the Enzymatic Reaction.

	$K_i \times 10^6$ M[a]	K_p[b]
19-Norprogesterone	0.5	63
17-Hydroxy-19-norprogesterone	2.3	1.0
19-Norandrostenedione	27	3.9
13β-Ethylgonenedione	18	8.6
19-Nortestosterone	13	0.9
17α-Methylnortestosterone	26	3.2
Nordienolone	10	0.7

[a] The K_i are determined in 3.3% methanol (v/v); see also footnote *a* of Table II. [b] See also Table III (footnote *a*), except that for 13β-ethylgonenedione the optical density is read at 310 mμ.

When one uses *different substrates or inhibitors*, there is correlation between partition coefficient and K_m or K_i when considering pregnene steroids on one hand and androstene and estrene steroids on the other hand. However within the last group the correlation is rather weak with the four inhibitors (see Table IV), and moreover 17-hydroxy-19-norprogesterone does not follow even the group "crude" rule. Actually it is expected that varying chemical groups on the steroid molecule not only changes its polarity, but also alters the hydrogen binding and electrostatic interactions which it can have with the enzymatic protein, and therefore interferes also with the chemical mechanism of the reaction.

With respect to the last consideration, K_m and K_i differences between various steroids and their 19-nor analogs could be explained on the following basis. Some of the factors influencing the rate of formation and stability of the enol form of Δ4-3-oxo steroids have been investigated by Subrahmanyam *et al.* (1966). A five- to sixfold enhanced rate of enol formation has been shown when comparing 19-norandrostenedione with androstenedione and was interpreted in terms of steric impediment by the C_{19} angular methyl group. The mechanism of the interaction of competitive inhibitors with isomerase has been obtained from fluorescence and absorption spectra and from isotopes experiments, which suggest formation of an enol as intermediate in the enzymatic reaction. If the enol form is effectively the active form combining with the enzyme, the two following reactions can be considered

$$\Delta 4\text{-3-oxo steroid} \longrightarrow \text{3-hydroxydiene steroid} \quad (1)$$

$$\text{3-hydroxydiene steroid} + \text{enzyme} \xrightarrow{k_i} \text{enzyme–3-hydroxydiene steroid} \quad (2)$$

If reaction 1 is limiting, which may be the case with the rather stable Δ4-3-oxo steroids, factors favoring enol formation will also increase formation of the enzyme–steroid complex and could explain the marked over-all affinity of the 19-nor derivatives for the enzyme.

Acknowledgments

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