transition state for the acylation reaction. In this way the conformational change induced by the S_{54}–P_{54} contact could play an important role in reducing the free energy of activation for the acylation reaction.

Summary

By studying the kinetics of hydrolysis of a variety of substrates which form only productive enzyme–substrate complexes, it has been possible to separate the roles of the various S_4–P_4 interactions. The region of S_4 (R) normally occupied by the α-methylene group of an amino acid side chain has been shown to bind α-methyl groups with about 1 kcal/mol. S_4R therefore contributes to substrate binding; it does not appear to significantly influence rates of reaction. In contrast, the region of S_4 (A) which would normally be occupied by the NH group of a P_4 amino acid residue binds α-amino, α-acetamido, and α-methyl groups reluctantly, but this occupation greatly increases the rate of bond cleavage. Higher rates of substrate hydrolysis are therefore accompanied by poorer enzyme–substrate binding. This finding is consistent with the postulated role of a conformational change in the catalytic process.

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Micellar Aggregation of Δ^5-3-Keto Steroids Lacking a Polar C-17 Group and Its Relation to the Activity and Specificity of the $\Delta^5 \rightarrow \Delta^4$-3-Ketosteroid Isomerase of *Pseudomonas testosteroni*†

J. Bryan Jones* and Keith D. Gordon‡

ABSTRACT: A systematic study has been carried out on the activity and apparent specificity of the $\Delta^5 \rightarrow \Delta^4$-3-ketosteroid isomerase of *Pseudomonas testosteroni* with respect to potential substrates possessing hydrophobic C-17β substituents. The series of Δ^5-3-ketones with the C-17 groups H, CH_3, CH_2CH_3, $CH(CH_3)_2$, and C_8H_{17}, of graded hydrocarbon character have been evaluated and the results obtained have confirmed that micellar aggregation of hydrophobic potential substrates in aqueous solutions is the main reason for the inability of the enzyme to catalyze their isomerization under the standard assay conditions. Under conditions which ensure significant disaggregation of the micelles, each compound studied became a good substrate of the isomerase. The specificity effects attributable to the C-17β groups were minor although the presence of the large C_8H_{17} group did result in significantly lower isomerization rates. An acid-catalyzed isomerization kinetic method was used to monitor the formation and disaggregation of the steriod micelles. This technique was found to be much more sensitive than the more traditional methods such as, for example, that based on light scattering. The effectiveness of various organic solvents, and of Tween-80 and Brij-35, in solubilizing the steroids was surveyed. Of the methods evaluated, the use of methanol as cosolvent provided the best compromise for achieving a maximum of steroid solvation with a minimum of enzyme inactivation. The data obtained indicate that a C-17–ring D region binding locus of hydrophobic character is present at the active site and that binding of C-17 hydrophobic Δ^5-3-ketones in the ES complex helps to maintain the conformation of the active-site region against the inactivating effects of up to 30% methanol. Furthermore, it has been found that only a small proportion of the hydrophobic substrates needs to be solubilized for complete and rapid enzymic isomerization to take place since the solvated molecules and those present in the substrate micelles undergo facile equilibration.

The Δ^5-3-ketosteroid isomerase of *Pseudomonas testosteroni* (EC 5.3.3.1) is one of the most active enzymes known and many aspects of its capacity to isomerize a broad spectrum of steroid-5-en-3-ones to the corresponding Δ^4-3-ketones have been studied (Talalay, 1965; Sih and Whitlock, 1968; Malhotra and Ringold, 1965; Falcoz-Kelly *et al.*, 1968; Jones

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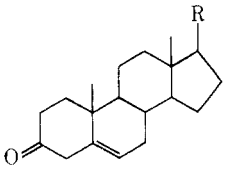
‡ National Research Council of Canada Scholarship Holder, 1968–1971.

and Wigfield, 1968, 1969; Weintraub *et al.*, 1972). Our previous studies had indicated that Δ^5-3-keto steroids with nonpolar C-17 substituents, such as androst-5-en-3-one (**1a**) and



a, R = H
b, R = CH_3
c, R = CH_2CH_3
d, R = $CH(CH_3)_2$
e, R = C_8H_{17}
f, R = O
g, R = $COCH_3$

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cholest-5-en-3-one (**1e**), were not substrates of the enzyme due to their micellar aggregation in aqueous solvents (Jones and Wigfield, 1968). However, the data suggested that if disaggregation to the fully solvated species could be effected, such molecules would be efficiently isomerized by the enzyme. These observations prompted us to undertake a systematic study of the influence of nonpolar C-17 substituents on the aggregation tendencies of such Δ^5-3-ketones and to evaluate their ease of isomerization by the enzyme under conditions ensuring that a significant proportion of the monomeric form of each compound was present. The series of potential substrates **1a–e** possessing C-17β groups of graded hydrocarbon character was considered first and the conditions under which complete disruption of the micelles occurred were established using both light-scattering and acid-catalyzed isomerization procedures (Jones and Gordon, 1972a). This communication reports an extension of the latter study and applications of the data so obtained in further delineating the activity and C-17 specificity of the isomerase. In addition, information on the influence of a hydrophobic ring D region on enzyme–substrate binding has been obtained.

Materials and Methods

All materials and solvents used, including the Δ^5-3-ketones **1a–g**, were obtained and purified as described previously (Jones and Wigfield, 1968; Jones and Gordon, 1972b). Methanolic stock solutions of each steroid (0.24 and 1.5 mM) were used except for the experiments involving 1-propanol and tetrahydrofuran. In the latter studies stock solutions of the above concentrations were made up in the particular solvent being evaluated.

General Kinetic Procedures. The standard spectrophotometric assay procedure (Jarabak *et al.*, 1969; Jones and Wigfield, 1968) was used in all the kinetic determinations. Isomerizations were performed in either 3-ml, 1.0-cm path-length or 10-ml, 10-cm path-length thermostatted (at 25°) silica cells using a Cary 16 spectrophotometer. All kinetic data were subjected to least-squares regression analysis and correlation coefficients of >0.99 were obtained in all cases. All runs were carried out at least in duplicate and were reproducible to within $<\pm 3\%$ for the acid-catalysis experiments and $<\pm 10\%$ for the enzymic isomerizations. The enzyme solutions were prepared by up to 10^5-fold dilution of three-times-crystallized isomerase of specific activity 64,300 units/mg of protein with 1% bovine serum albumin of pH 7.0 and 0.01- to 0.05-ml aliquots of appropriate enzyme solutions were employed for each individual run. For each set of isomerizations the use of enzyme solutions of constant activity was ensured by assaying the isomerase concentrations by the standard procedure (Jarabak *et al.*, 1969). All rates were corrected for the uncatalyzed reaction at pH 7.0 and for the effect of the 1% bovine serum albumin in which the enzyme was dissolved (*cf.* Jones and Wigfield, 1968).

Comparison of the Effects of Methanol, 1-Propanol, and Tetrahydrofuran on the Enzyme-Catalyzed Isomerization of Androst-5-ene-3,17-dione (**1f**). The rates of enzymic isomerization of 25 μM solutions of androst-5-ene-3,17-dione in 0.03 M potassium phosphate buffer (pH 7.0) containing 1.6, 5, and 10%, respectively, of each of methanol, 1-propanol, and tetrahydrofuran were determined at 25° on 3-ml reaction volumes in 1-cm path-length cells.

Isomerizations of Cholest-5-en-3-one (**1e**) *in the Presence of Tween-80 and Brij-35.* Isomerizations of 25 μM solutions of cholest-5-en-3-one (**1e**) containing 1.6% methanol and 0.02–1.0% (v/v) of Tween-80 or Brij-35 were surveyed at 25° using the 1-cm path-length cells. Acid-catalyzed isomerizations were effected with 0.2 M KCl–HCl buffer (pH 1.0) and the attempted isomerase-catalyzed reactions in 0.03 M potassium phosphate buffer (pH 7.0).

Acid-Catalyzed Isomerizations of 1.2 μM Solutions of the Δ^5-3-Keto Steroids **1a–e**. All reactions were performed at 25° as described previously (Jones and Gordon, 1972a) using 10-cm path-length cells. The reaction mixtures were composed of 0.2 M KCl–HCl buffer of pH 1.0 [$(9.95 - x)$ ml], methanol (x ml), and 0.05 ml of the appropriate 0.24 mM methanolic stock solution of the steroids **1a–e**. The minimum proportion of methanol required for the isomerization to show a pseudo-first-order dependence on steroid concentration, and the corresponding rate constants, were then determined for each of **1a–e**.

Enzyme-Catalyzed Isomerizations of 1.2 μM Solutions of the Δ^5-3-Keto Steroids **1a–f**. The general enzyme assay procedure (Jarabak *et al.*, 1969; Jones and Wigfield, 1968) was applied using 10-cm path-length cells and reaction mixtures were made up of 0.03 M potassium phosphate buffer, pH 7.0 [$(9.9 - x)$ ml], methanol (x ml), enzyme solution (0.05 ml), and 0.05 ml of an 0.24 mM methanolic stock solution of one of **1a–f**. The net enzymic isomerization rates were obtained by subtracting the components arising from catalysis by bovine serum albumin alone. The latter were determined in an identical manner by substituting 1% bovine serum albumin (pH 7.0) for the enzyme solution in the assay mixture.

Determination of K_m and V_{max} for the Enzyme-Catalyzed Isomerization of Androst-5-en-3-one (**1a**). The isomerizations were studied at 25° in 12.5% methanol in the 10-cm path-length cells containing 0.03 M phosphate buffer, pH 7.0 (8.73 ml), enzyme solution (0.02 ml), methanol ($(1.25 - x)$ ml), and 0.24 mM methanolic steroid stock solution (x ml). The substrate concentration was varied from 0.48 to 1.47 μM. The data were then treated according to the standard procedure of Lineweaver and Burk (1934).

Results

Earlier investigations (Jones and Gordon, 1972a) on the series of Δ^5-3-ketones **1a–e** possessing C-17 groups of progressively increasing hydrophobic character had delineated some of the factors involved in their tendencies to form micelles in aqueous solutions and had shown that of the various organic solvents surveyed, 1-propanol and tetrahydrofuran were the most effective in achieving disruption of the aggregates. However, it did not prove possible to capitalize on the superior substrate solvating capabilities of the latter cosolvents for the intended enzyme specificity studies since, as Figure 1 shows, it was found that their addition to isomerase-containing solutions resulted in significant inactivation of the

TABLE I: Acid-Catalyzed Isomerizations of Cholest-5-en-3-one (**1e**) in Aqueous Solutions Containing Tween-80.[a]

% Tween-80 (v/v)	k ($sec^{-1} \times 10^4$)
1.0	0.95
0.04	1.01
0.02	1.45

[a] The rate constants were calculated from data obtained at 25° on 25 μM solutions of steroid using 0.2 M KCl–HCl buffer (pH 1.0) as the catalyst.

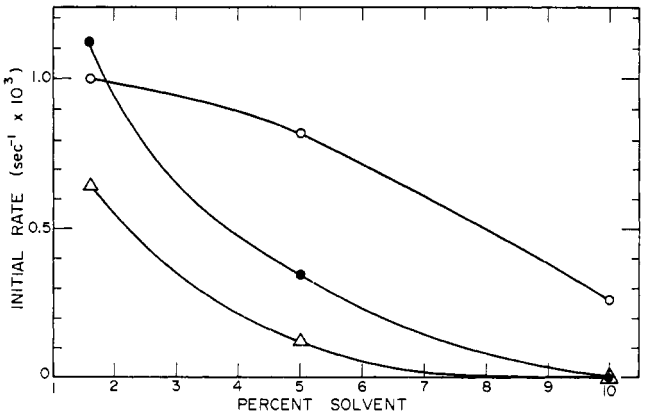

FIGURE 1: Effect of the addition of methanol (○), tetrahydrofuran (●), and 1-propanol (△) on the activity of the isomerase. The assays were performed in 0.03 M potassium phosphate buffer (pH 7.0) at 25° on 25 μM solutions of androst-5-ene-3,17-dione (**1f**).

enzyme. In contrast, the effects of adding equivalent amounts of methanol, the cosolvent most often used in isomerase studies (Talalay, 1965; Falcoz-Kelley *et al.*, 1968; Jones and Wigfield, 1968) were much less dramatic. Under the normal assay conditions, using 25 μM solutions of the standard substrate androst-5-ene-3,17-dione (**1f**), the loss of enzymic activity was virtually complete in aqueous solutions containing 10% of either 1-propanol or tetrahydrofuran. Since such concentrations of these organic cosolvents were still well below those required for complete solvation of **1a–e** under the conditions surveyed, attention was turned toward alternative methods of disrupting the steroid micelles.

In view of the report (Reynier *et al.*, 1969) that enzymic catalysis of steroid reactions is possible within the environment of a nonionic surfactant micelle, the possibility of studying the isomerization of **1a–e** by the *P. testosteroni* enzyme under such conditions was considered. The effects of Tween-80 on 25 μM aqueous solutions of cholest-5-en-3-one (**1e**) were surveyed initially using the acid-catalysis isomerization technique (Jones and Gordon, 1972a) to monitor the solubilization process. The isomerization was found to follow pseudo-first-order kinetics in 1.0% (v/v) aqueous solutions of Tween-80, thus demonstrating that disruption of the **1e** micelles was complete under such conditions. Similar results were still obtained when the Tween-80 proportion was reduced to 0.02% (v/v). As Table I shows, the first-order rate constants for the isomerization increase as the concentration of the surfactant is lowered. In contrast, in the presence of similar concentrations of Brij-35, **1e** was not fully solubilized as evidenced by the multiple-order dependences on steroid concentration observed for acid-catalyzed isomerizations carried out under analogous conditions. Disappointingly, no isomerization of **1e** occurred when 0.02–1.0% (v/v) of Tween-80 or Brij-35 were added to the normal enzyme assay solution nor when the isomerase concentration was increased by 20-fold.

At this stage we reexamined the data available on the relative capacities of various organic solvents to solvate the hydrophobic Δ^5-3-ketones in aqueous solutions and on the influence of such solvents on the activity of the Δ^5-3-ketoisomerase (Jones and Wigfield, 1968; Falcoz-Kelly *et al.*, 1968; Weintraub *et al.*, 1972; Jones and Gordon, 1972a). It soon became evident that methanol provided the best overall balance of properties for effecting solubilization of steroids such as **1a–e** while allowing the enzyme to retain maximal activity. However, the 27–75% methanol levels required to completely disaggregate micelles of each of **1a–e** under the normal assay conditions (*viz.*, using 25 μM substrate solutions) were too high to permit a realistic comparison of the compounds as potential substrates of the isomerase. Accordingly, the effect of lowering the steroid concentration on the critical methanol concentration was evaluated. The minimum methanol concentrations required to ensure the absence of aggregates in 1.2 μM aqueous solutions of each of **1a–e** were again determined by the acid–catalysis isomerization technique. The results obtained (Table II) show that, as expected, significant decreases in the critical methanol concentrations are observed on lowering the steroid concentrations from 25 to 1.2 μM. The kinetics observed again followed the characteristic pattern of multiple-order dependence on steroid concentration while micelles were present with the reaction order becoming unity when the methanol concentration reached the minimum level at which each particular Δ^5-3-ketone was fully solvated. The fact that, when pseudo-first-order kinetics are ensured, the rate constants for each of the steroids **1a–e** are approximately equal shows that no significant long-range effect is exerted on the isomerization process by the C-17 group.

The critical methanol concentrations recorded in Table II are higher than those used in most of the previous specificity and mechanistic studies carried out on the Δ^5-3-ketoisomerase (Kawahara *et al.*, 1962; Nes *et al.*, 1963; Malhotra and Ringold, 1965; Falcoz-Kelly *et al.*, 1968; Jones and Wigfield, 1968). Nevertheless, we decided to examine the isomerase-catalyzed transformations of 1.2 μM methanolic solutions of **1a–e**, and, for reference, of the standard substrate **1f**, since this steroid level represented the limit of our current experimental capacity to monitor the process. The results obtained, which are summarized in Table III, show clearly that considerations of the aggregation tendencies and hydrophobic properties of Δ^5-3-keto steroids lacking a C-17 polar group are of overriding importance with respect to their behavior as substrates. (Although the catalytic efficiency of the isomerase was markedly diminished in the solutions of higher methanol content (*cf.* Falcoz-Kelly *et al.* (1968), the residual activity of the enzyme remained constant well beyond the 3- to 5-min period required for the kinetic measurements and the initial activity was restored on dilution (*cf.* Weintraub *et al.*, 1972).) The data of Table III indicate that, under the same assay conditions, the substrates with hydrophobic C-17–ring D regions are more tightly bound to the active site than those, such as **1f**, with polar C-17 functions. Further support for this conclusion was provided by comparing the K_m values of **1f**, **1g**, and **1a**, for which series the C-17 region becomes

TABLE II: Acid-Catalyzed Isomerizations of 1.2 μM Solutions of **1a–e** in Aqueous Methanol.[a]

	1a		**1b**		**1c**		**1d**		**1e**	
% Methanol ()[b]	React. Order[c]	Rate Constant[d]	React. Order	Rate Constant	React. Order	Rate Constant	React. Order	Rate Constant	React. Order	Rate Constant
12.5 (27)	1	1.40	m[e]		m		m		m	
22.0 (30)	1	1.23	1	1.25	m		m		m	
30.0 (35)	1	1.09	1	1.02	1	1.10	m		m	
35.0 (50)	1	0.97	1	0.95	1	1.01	1	0.95	m	
50.0 (75)	1	0.64	1	0.60	1	0.61	1	0.64	1	0.63

[a] Isomerizations were effected at 25° with 10-ml reaction volumes in 10-cm path-length uv cells using 0.2 M KCl–HCl buffer (pH 1.0) as the catalyst. All runs were carried out at least in duplicate and the data were reproducible to $<\pm 3\%$. All data were subjected to least-squares regression analysis and correlation coefficients of >0.99 were obtained in all cases. [b] Corresponding minimum % methanol required for 25 μM solutions (Jones and Gordon, 1972a). [c] With respect to steroid concentration. [d] In $sec^{-1} \times 10^3$. [e] Multiple order.

progressively less polar, under conditions ensuring that each substrate was fully solvated. As Table IV shows, the K_m values in 12.5–13% aqueous methanol decrease markedly as the C-17 region becomes more hydrophobic.

Discussion

The initial attempts to define conditions which would enable each of **1a–e** to be sufficiently solvated to permit further delineation of the C-17 specificity of the $\Delta^5 \rightarrow \Delta^4$-3-keto-isomerase of *P. testosteroni* were centered on the use of organic solvents to disrupt the steroid micelles which formed in aqueous solution. Although the isomerase is peculiarly tolerant of relatively high concentrations of several organic solvents (Jones and Wigfield, 1968; Falcoz-Kelly *et al.*, 1968; Weintraub *et al.*, 1972), it was obviously desirable to carry out any enzymic studies in aqueous solutions containing the smallest feasible proportion of cosolvent. Of the organic solvents which had been evaluated those effecting complete solvation of **1a–e** in the lowest concentrations were 1-propanol and tetrahydrofuran (Jones and Gordon, 1972a). Even though the hydrophobic interactions involved in maintaining micelle and protein structures are often similar in nature (Mukerjee, 1967; Emerson and Holzer, 1967), it had been hoped that the addition of the comparatively small proportions of these solvents required to disaggregate **1a–e** in aqueous solution would not have too adverse an effect on the isomerase. However, the decrease in enzymic activity was found (Figure 1) to parallel the micellar disaggregation power of the solvent. These results are in agreement with the more extensive data of Weintraub *et al.* (1972) on the effects of 1-propanol and other solvents on the binding and catalytic properties of the isomerase.

The abilities of nonionic surfactants to solubilize steroids (Elworthy *et al.*, 1968; Sjöblöm, 1967) in aqueous solutions, coupled with a favorable literature (Reynier *et al.*, 1969) prognosis for enzymic catalysis within such systems, prompted our consideration of the representative nonionic surfactants Tween-80 and Brij-35 for this purpose. Unfortunately, although Tween-80 was shown by the acid–catalysis method to be very effective in solubilizing the most hydrophobic of our Δ^5-3-ketones, **1e** (Table I), no corresponding enzymic isomerization of **1e** could be induced in its presence. Under the same range of conditions, complete disruption of the aggregates of **1e** did not occur in aqueous solutions of Brij-35 and again, no enzymic catalysis could be induced by the addition of the surfactant to the assay solutions.

A satisfactory solution to the above dilemmas was finally provided by using more dilute steroid solutions in order to

TABLE III: Enzyme-Catalyzed Isomerizations of 1.2 μM Solutions of **1a–f** in Aqueous Methanol.[a]

	Net Enzymic Rate Constants ($sec^{-1} \times 10^4$)[b]					
% MeOH	**1a**	**1b**	**1c**	**1d**	**1e**	**1f**
1.5	6.3	4.2	2.1	1.2	<0.01	14.3
12.5	4.8	5.4	3.4	2.1	2.3	1.8
22.0	2.8	3.8	2.5	2.4	0.1	<0.01
30.0	1.0	1.5	1.2	2.0	<0.01	<0.01

[a] Isomerizations were carried out at 25° in 10-ml volume, 10-cm path-length cells. The assay solutions were made up of 0.03 M potassium phosphate buffer (pH 7.0) containing varying amounts of methanol to which appropriate aliquots of steroid, and subsequently enzyme, stock solutions were added. The same enzyme concentration was used for each run. Duplicate experiments, which were reproducible to $\leq \pm 10\%$, were carried out and all data were subjected to least-squares regression analysis. [b] Calculated from the initial rates of isomerization and corrected for catalysis by 1% bovine serum albumin alone.

TABLE IV: K_m and V_{max} Values for the Enzyme-Catalyzed Isomerizations of Androst-5-en-3-one (**1a**), Androst-5-ene-3,17-dione (**1f**), and Pregn-5-ene-3,20-dione (**1g**).

Substrate	% MeOH	K_m (M $\times 10^5$)	V_{max} (mol (S) sec^{-1} mol $(E)^{-1} \times 10^4$)
1a	12.5	0.5	0.75
1g[a]	13	9.0	11.0
1f[a]	13	91.0	9.3

[a] Falcoz-Kelly *et al.* (1968).

enable the proportions of cosolvent required to achieve complete solvation of the substrate to be reduced to levels tolerated by the isomerase. Methanol was used exclusively as the cosolvent in all the studies carried out on the lower steroid concentration solutions since, although it was not the most effective in causing micelle breakup, its influence on the enzyme's activity was less unfavorable than for any of the other co-solvents investigated (Weintraub *et al.*, 1972). Accordingly, the effect of reducing the concentrations of **1a–e** from the normal 25 μM level to 1.2 μM on the quantities of methanol required for complete solvation of the steroids was studied, again using the acid-catalysis isomerization method to monitor the disaggregation process. The ability of this method to detect the onset of micelle formation in such dilute steroid solutions demonstrates the extreme sensitivity of the technique. We were unable to follow the disaggregation process at these low steroid concentrations using the more conventional light-scattering method and it seems unlikely that any of the other physicochemical methods normally used (Shinoda *et al.*, 1963; Fendler and Fendler, 1970) in micelle studies would be effective for solutions containing so little of the steroids. As had been anticipated, significant reductions were observed (Table II) in the proportions of methanol needed to ensure complete solvation of the steroids in the diluted solutions. Although the minimum methanol levels required were still somewhat higher than desirable for enzymic studies, they were not outside the range known to be tolerated by the isomerase (Jones and Wigfield, 1968; Falcoz-Kelly *et al.*, 1968; Weintraub *et al.*, 1972).

The molecules within a micelle are known to be in rapid equilibrium with those in free solution and in other micelles (Mukerjee, 1967; Hofmann and Small, 1967). Thus even at concentrations of methanol lower than those required for complete micelle breakup, it was anticipated that significant amounts of the monomeric forms of the steroids **1a–e** would often be present. Furthermore, since the proportion of solvated monomer should be inversely related to the aggregation tendency, it was felt that conducting the enzymic isomerizations in solutions of both low and high methanol contents should enable an estimate to be made of the importance of substrate aggregation with respect to the apparent specificity and activity of the enzyme towards hydrophobic substrates.

The enzymic isomerization data (Table III) for 1.2 μM methanolic solutions of **1a–e** and of the standard substrate androst-5-ene-3,17-dione (**1f**) confirm that under the normal assay conditions the apparent specificity of the isomerase against Δ^5-3-ketones lacking a polar C-17 group is mainly due to their aggregation in the dilute aqueous methanol solutions used. In 1.5% aqueous methanol, the increasing tendency to form micelles as the C-17 substituent changes from H through C_8H_{17} in the series **1a–e** is paralleled by a decreasing rate constant trend for the enzymic isomerization. As the hydrophobic character of the C-17 group becomes more dominant, the amount of fully solvated substrate present decreases progressively until for cholest-5-en-3-one (**1e**) no significant quantity of the monomeric substrate is present even in a solution of such low steroid concentration. That the rate differences in 1.5% methanol did not reflect a true specificity effect was verified by the data obtained in 12.5% aqueous methanol in which solvent the proportions of solvated **1a–e** were sufficient to enable each one to become a better substrate than the standard, **1f**. (Even though the overall effects of methanol on the enzymic isomerizations of different substrates cannot be predicted with any assurance (*cf.* Falcoz-Kelly *et al.*, 1968; Weintraub *et al.*, 1972), qualitative or semiquantitative interpretations of rate differences in the same solvent composition are considered valid.) The rate constant variations in 12.5, 22, and 30% aqueous methanol, respectively, demonstrate that the isomerase is influenced by the nature of the C-17β groups of **1a–e**. However, except in the case of **1e**, where the large C_8H_{17} side chain is present, the specificity effect appears to be a minor one. The facilities with which **1a–e** are isomerized when only small proportions of monomeric steroids are present support the view that the solvated substrate, and presumably product, molecules equilibrate rapidly with those within the aggregates under the assay conditions.

The increasing inactivation of the isomerase resulting from the progressive addition of methanol is reflected in the decreasing isomerization rates for androst-5-ene-3,17-dione (**1f**), for which the enzyme-catalyzed reaction is negligible in 20% aqueous methanol (*cf.* Falcoz-Kelly *et al.*, 1968; Weintraub *et al.*, 1972). In contrast, except for cholest-5-en-3-one, the isomerization rates for the series **1a–e** are significant even in 30% aqueous methanol. These results indicate that, other than in the region of the ring A binding locus (Talalay, 1965; Malhotra and Ringold, 1965; Sih and Whitlock, 1967; Jones and Wigfield, 1969; Jones and Ship, 1972), the active site of the enzyme is largely nonpolar in character and that formation of the ES complex with hydrophobic substrates protects, at least partially, the active-site region of the isomerase against the inactivating effects of the higher methanol content solutions. The Δ^5-3-ketones with the smaller C-17β alkyl groups are the most effective in this regard (Table III). The total data are consistent with a C-17–ring D region binding locus of hydrophobic character being present at the active site. From the current and previous specificity studies (Kawahara *et al.*, 1962; Nes *et al.*, 1963; Alfsen *et al.*, 1966; Jones and Wigfield, 1968; Falcoz-Kelly *et al.*, 1968) it is evident that the locus favors C-17β substituents and that C-17α functions interfere with binding at this site.

Further evidence substantiating the conclusion that interactions of hydrophobic ring D substrate regions with their corresponding active-site locus can make significant contributions to the strength of ES binding was provided by a comparison of the kinetic constants for the enzymic isomerizations of **1f**, **1g**, and **1a** possessing the C-17 substituents, = O, $COCH_3$, and H, respectively, of decreasing polarity. As Table IV shows, the K_m values diminish in the order **1a** < **1g** < **1f**. Since for these substrates K_m is considered to approximate very closely the dissociation constant of the ES complex,[1] it is clear that the less polar the C-17 group the more tightly bound is the substrate at the active site. When the fact that the assay solution contained 12.5% of methanol is taken into account, the K_m value for androst-5-en-3-one (**1a**) of 0.5×10^{-5} M is an extremely low one and **1a** is considered to be the best-binding substrate for which quantitative data are currently available. However, its potential as an excellent substrate is somewhat offset by its relatively low V_{max} value. In view of the even higher methanol concentrations required to achieve

[1]This follows from the observations (Falcoz-Kelly *et al.*, 1968; Sih and Whitlock, 1968; H. J. Ringold, personal communication) that the K_m's for **1g,f** are very similar to the K_i values for the corresponding Δ^4-3-ketone isomerization products. For this enzyme, the structures of, and binding interactions involved in, the ES and EP complexes will be virtually identical. Thus since the K_i of a Δ^4-3-ketone is a true dissociation constant, the K_m of the precursor Δ^5-substrate must approximate the dissociation constant of ES and is an accurate measure of enzyme–substrate affinity. It may be noted that the above analysis applies to *any* isomerase and such enzymes are therefore particularly well suited to quantitative substrate binding studies by the steady-state method.

complete solubilization of the remaining C-17 alkylated Δ^5-3-ketones of the series, the K_m and V_{max} determinations were not extended to the more hydrophobic substrates **1b–e**.

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Cytochrome P-450 in 7α-Hydroxylation of Taurodeoxycholic Acid†

Dietmar Trülzsch, Helmut Greim, Peter Czygan, Ferenc Hutterer, Fenton Schaffner, Hans Popper, David Y. Cooper, and Otto Rosenthal

ABSTRACT: Taurodeoxycholic acid is 7α hydroxylated to form taurocholic acid by rat liver microsomes in the presence of NADPH. This enzymatic reaction has a K_m of 0.03 mM. The reaction is inhibited by $CO:O_2$ mixtures with a K (Warburg's partition constant) of 0.5. The inhibition is maximally reversed by monochromatic light at the wavelength of 450 nm. These observations establish the P-450 dependence of 7α hydroxylation of taurodeoxycholic acid.

The strongly detergent chenodeoxycholic acid is converted to the weakly detergent β-muricholic acid by liver enzymes in the rat (Greim *et al.*, 1972). This conversion involves 6β hydroxylation which has been shown to be cytochrome P-450 dependent (Voigt *et al.*, 1968). The purpose of this study was to determine if the 7α hydroxylation of deoxycholic acid to yield cholic acid likewise requires cytochrome P-450.

The presence of cholic acid was first demonstrated in the bile of bile fistula rats by Bergström and coworkers (Bergström *et al.*, 1953). More recently Einarsson and Johansson (1968) have found that this reaction is catalyzed by enzymes localized in the microsomal fraction of the liver homogenate and requires NADPH and molecular O_2. Furthermore these authors showed that 7α-hydroxylase activity is induced by pretreatment of the rats with phenobarbital. All of these properties suggest that this hydroxylation is catalyzed by cytochrome P-450 (Cooper *et al.*, 1965). To establish the role of P-450 in the deoxycholic acid hydroxylation we have studied the CO inhibition and the efficiency of various wavelengths of monochromatic light of equal intensity, relative to that at 450 nm, in reversing this CO inhibition.

Materials and Methods

Chemicals were obtained from the following sources: sodium salts of taurodeoxycholic acid (3α,12α-dihydroxy-5β-

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