Measurements of H^3 and C^{14}. All radioactive counting was done in Packard Tri-Carb scintillation spectrometers: at Milstead Laboratory in a Model 314EX, and at the Worcester Foundation in a Model 3002. Both laboratories used the same scintillator solution (4 g. of 2,5-diphenyloxazole and 100 mg. of 1,4-bis-2-(5-phenyloxazolyl)benzene per l. of toluene). Reference standard solutions of the benzhydrylamide of the H^3-C^{14}-mevalonate and of the squalene and cholesterol biosynthesized from it were counted at both laboratories: the H^3/C^{14} ratios found at the Worcester Foundation were consistently higher by a factor of 8.12/7.37 than those measured at Milstead. Since further experimental results obtained with the same specimen of H^3-C^{14}-mevalonate as used here await publication from Milstead Laboratory, the data of radioactive assays from the Worcester Foundation have been all multiplied by the factor 7.37/8.12.

Chemistry of Conjugate Anions and Enols. V. Stereochemistry, Kinetics, and Mechanism of the Acid- and Enzymatic-Catalyzed Isomerization of ^{5}-3-Keto Steroids[1,2]

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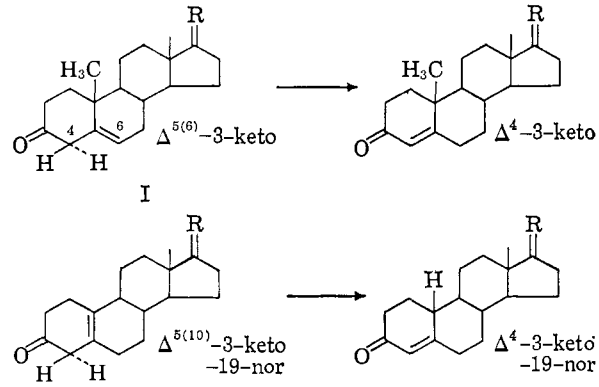
Contribution from the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts. *Received March 12, 1965*

The acid-catalyzed isomerization of Δ^5-3-keto steroids has been shown to proceed by formation of the $\Delta^{3,5}$-dienol via a rate-determining loss of a C-4 proton. The kinetic isotope effect (k_H/k_D = 4.1) and the solvent isotope effect (k_{D_2O}/k_{H_2O} = 1.64) rule out a mechanism involving C-6 protonation of the double bond followed by C-4 proton loss. Loss of the 4α-proton was shown to be slightly favored over 4β-proton loss in the enolization process. The enzymatic reaction with Δ^5-3-ketoisomerase was found to involve a stereospecific diaxial proton transfer from the 4β- to the 6β-position and was shown to be intramolecular by the simultaneous isomerization of deuterated and nondeuterated substrates. The loss of the 4β-proton was found to be rate determining in the enzymic reaction V_{max_H}/V_{max_D} = 5.35, k_{m_H} = 3.1 × 10^{-4}, K_{m_D} = 1.4 × 10^{-4}. It is proposed that the Δ^5-3-ketone undergoes conversion to the enol through carbonyl protonation by a donor group (AH) on the enzyme followed by 4β-proton loss to a basic group (B). In a fast step, reprotonation at the 6β-position by BH and proton removal from the carbonyl by A forms the Δ^4-3-ketone and regenerates the enzyme. The kinetics of the reaction and the failure of BH to undergo proton exchange with the medium are discussed.

Introduction

In 1955 Talalay and Wang[4] isolated an induced enzyme from *Pseudomonas testosteroni* that catalyzes the isomerization of a number of $\Delta^{5(6)}$-3-keto steroids and of $\Delta^{5(10)}$-19-nor steroids to the corresponding α,β-unsaturated ketone. Since the enzyme requires no cofactor and the over-all reaction is an extremely simple one, the isomerase reaction appeared particularly attractive for a study of stereochemistry, kinetics, and mechanism. Apart from its simplicity the enzymic reaction is characterized by two points of exceptional interest, the first being the extremely high turnover number of 17 × 10^6 min.$^{-1}$ (at maximum velocity one mole of enzyme isomerizes 17 × 10^6 moles of androst-5-ene-3,17-dione (I, R = ketone) per minute), which classifies this purified crystalline enzyme as the most active known.[5] Furthermore, it had been demonstrated[4,5] that enzymatic isomerization carried out in deuterium oxide led to the incorporation of only 0.12 atom of deuterium into the product, indicating that the net process involved a transfer of hydrogen from the C-4 position of the β,γ-unsaturated ketone to the C-6 position of the resulting α,β-unsaturated ketone product. In contrast, both the acid- and base-catalyzed isomerization led to the incorporation of one or more atoms of deuterium into the product.



In this paper we report on the mechanism and stereochemistry of both the acid-catalyzed and the enzymatic isomerization process. Apart from the obvious interest as a model for the enzymatic reaction, the chemical reaction constitutes a study which clarifies certain fundamental points concerned with the chemistry of

(1) This work was supported by the National Institutes of Health Research Grant No. AM-4044 and the American Cancer Society Grant T-185.

(2) Presented in part at the 6th International Congress of Biochemistry, New York, N. Y., July 1964, Abstracts IV, p. 139.

(3) To whom inquiries should be addressed.

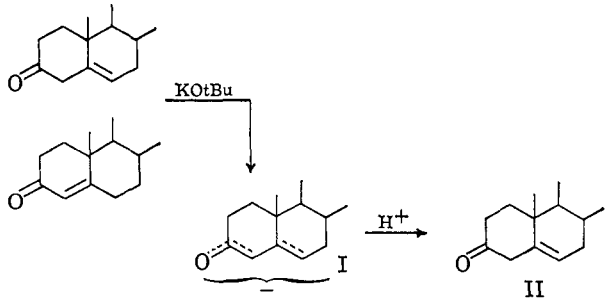
(4) P. Talalay and V. S. Wang, *Biochim. Biophys. Acta*, **18**, 300 (1955).

(5) F. S. Kawahara and P. Talalay, *J. Biol. Chem.*, **235**, 1, 1960; F. S. Kawahara, S. F. Wang, and P. Talalay, *ibid.*, **237**, 1500 (1962); S. F. Wang, F. S. Kawahara, and P. Talalay, *ibid.*, **238**, 576 (1963).

conjugate enol and enolate formation and protonation and as such represents a continuation of our studies of this system.[6]

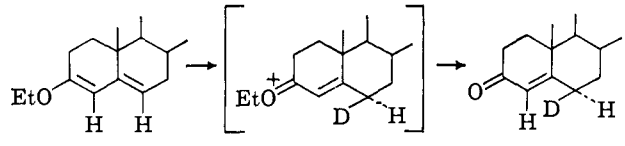
Acid-Catalyzed Isomerization

While it may have been anticipated *a priori* that acid isomerization of the β,γ-unsaturated ketone proceeded *via* an enolization mechanism (Path A, Figure 1) it became necessary to rigidly establish this point for reasons which become obvious from the following discussion and observations. The conjugate enolate anion (I), which may be generated from either the α,β- or β,γ-unsaturated ketone, undergoes with acid kinetically controlled C-4 protonation to form the thermodynamically unstable β,γ-unsaturated ketone II.[7] If, as has been commonly assumed, the enolate



anion undergoes protonation first on oxygen to generate the neutral enol, then acid protonation of the neutral enol and of the enolate anion should occur at the same (C-4) position.

Deuterium Incorporation. Enol Ether. To establish the position of protonation of the neutral enol the hydrolysis of an enol ether (3-ethoxycholesta-3,5-diene) was studied first as a mechanistic parallel. Hydrolysis



effected by deuterioacetic acid or deuterium chloride in diglyme–deuterium oxide led to the Δ^4-3-ketone containing an atom of deuterium at the axial 6β-position (C–D stretching frequency 2140 cm.$^{-1}$) but free of deuterium at C-4 as shown by analysis and by the absence of a characteristic C–D stretching frequency at 2255 cm.$^{-1}$ in the infrared.[6,8] Therefore it appeared that the enol ether underwent protonation exclusively at the γ-position (C-6) and not at C-4, in common with the attack of "positive" halogen or of peracid on the enol ether.

α,β-Unsaturated Ketone. Previous studies[6,8] demonstrated that the enolization of Δ^4-3-keto steroids with strong deuterated acid led to the preferential formation of the $\Delta^{3,5}$-dienol and to selective deuterium incorporation at C-6. Thus, when the α,β-unsaturated ketone, cholest-4-en-3-one, was treated with deuterium chloride in diglyme–deuterium oxide until 1.5 atoms of deuterium

(6) Previous paper in this series: S. K. Malhotra and H. J. Ringold, *J. Am. Chem. Soc.*, **86**, 1997 (1964).
(7) H. J. Ringold and S. K. Malhotra, *Tetrahedron Letters*, 669 (1962).
(8) S. K. Malhotra and H. J. Ringold, *J. Am. Chem. Soc.*, **85**, 1538 (1963).

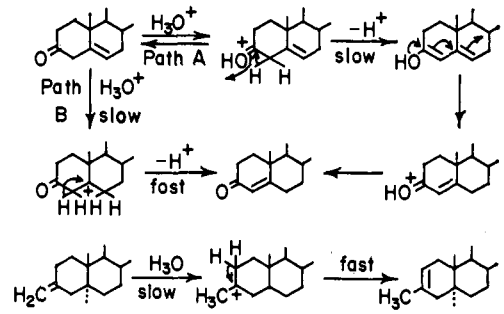
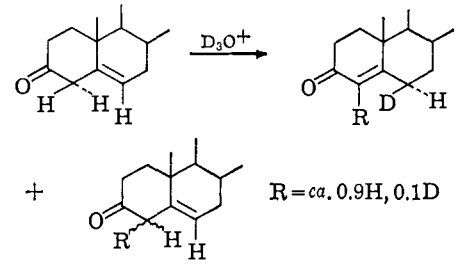


Figure 1.

had been incorporated, it was shown that essentially a full atom of deuterium had been introduced into the 6β-position while no more than 0.1 atom of deuterium appeared on the double bond at C-4. Essentially identical results were observed with another Δ^4-3-ketone, testosterone, as starting material.

β,γ-Unsaturated Ketone. When the β,γ-unsaturated ketone, cholest-5-en-3-one, was isomerized by brief treatment with deuterium chloride in diglyme–deuterium oxide, 6β-deuteriocholest-4-en-3-one was obtained containing less than 0.1 atom of deuterium at C-4. In an identical isomerization stopped at the 70% point the starting β,γ-unsaturated ketone was reisolated by thin layer chromatography and shown by infrared analysis not to contain any significant quantity of deuterium at C-4 (or elsewhere). Finally, when another β,γ-unsaturated ketone, 17β-hydroxyandrost-5-en-3-one, was isomerized as described above, and the reaction was stopped at the 50% point, the recovered β,γ-unsaturated ketone, which was frozen by immediate reduction with lithium aluminum hydride, contained only 0.08 atom of deuterium at C-4.



Mechanism of Isomerization of β,γ-Unsaturated Ketone. While these experiments strongly indicated that the neutral enol and the enol ether underwent preferential protonation at the C-6 (γ) position while the enolate anion protonated at C-4, another possibility existed if, in fact, the β,γ-unsaturated ketone did not isomerize *via* the enol. An alternate pathway was theoretically possible for this isomerization (path B, Figure 1), involving C-6 protonation of the double bond followed by loss of the C-4 proton. This pathway would not involve an enolic intermediate and would account for the failure to incorporate significant quantities of deuterium into the C-4 position of the unreacted β,γ-unsaturated ketone as well as into the C-4 position of the resulting α,β-unsaturated ketone. Although there could be no doubt that the $\Delta^{3,5}$-enol had been generated by acid treatment of the α,β-unsaturated ketone, there was still a possibility, however unlikely, that this enol underwent preferential protonation

Table I. Rates of Acid-Catalyzed Isomerization of Δ^5-3-Keto Steroids.[a] Isotope and Solvent Effects

Compd.	Medium	$k \times 10^3$, sec.$^{-1}$	k_H/k_D	k_{D_2O}/k_{H_2O}
Androst-5-ene-3,17-dione	0.12 *N* HCl–H_2O	2.19		
Androst-5-ene-3,17-dione	0.12 *N* DCl–D_2O[b]	3.41		1.56[b]
4β-Deuterioandrost-5-ene-3,17-dione[c]	0.12 *N* HCl–H_2O	1.45	1.51	
4,4-Dideuterioandrost-5-ene-3,17-dione[d]	0.12 *N* HCl–H_2O	0.53	4.14[d]	

[a] Conditions for these kinetic studies and isotope distribution of the starting materials are given in the Experimental section. [b] 88% D_2O–12% H_2O; k_{D_2O}/k_{H_2O} is uncorrected for the 12% water. [c] 0.89 atom of deuterium. [d] 1.84 atoms of deuterium; k_H/k_D is uncorrected for the nondeuterated species.

(deuteration) at the C-4 position to yield the β,γ-unsaturated ketone which was then rapidly reconverted to the α,β-unsaturated ketone by the alternate pathway not involving formation of the enol. To invoke this alternate pathway and still account for the relative lack of deuterium incorporation at C-4, it would be necessary that the theoretical alternate isomerization route be a much faster reaction than enolization of the β,γ-unsaturated ketone and further, that protonation of the neutral enol at C-4 be a highly stereospecific process paralleled by an equally stereospecific process in the isomerization step. The same arguments could also hold true in the hydrolysis of the

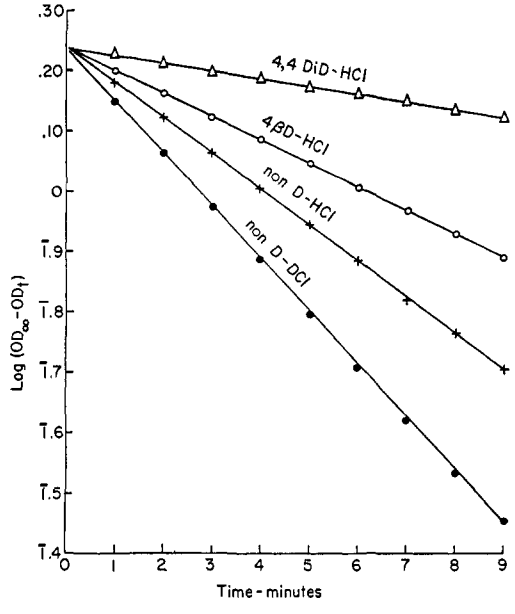


Figure 2. Kinetics of the acid-catalyzed isomerization of deuterated and nondeuterated androst-5-ene-3,17-dione.

enol ether. These restrictions, while considered to be highly unlikely, were not impossible; therefore further data was sought to definitely establish the enolization mechanism A or the double-bond protonation mechanism B. A precedent[9] for mechanism B is found, in fact, in the isomerization of 3-methylenecholestene to 3-methylcholest-2-ene (Figure 1), which has been shown to proceed by a slow protonation of the double bond followed by a rapid proton loss from C-2.

Kinetics and Stereochemistry. The enolization mechanism A was readily established on the basis of the isotope effects found with 4,4-dideuterated β,γ-unsaturated ketones and on the basis of solvent isotope effects. The enolization reaction, involving a slow rate-determining proton loss from C-4, should exhibit a substantial primary deuterium isotope effect. Also, enol formation and hence isomerization should proceed faster in strong deuterated acid than in strong protiated acid due to the relatively higher steady-state concentration[10] of the O-deuteronated ketone. In contrast, isomerization *via* mechanism B, with a slow rate-determining protonation of the double bond, should proceed without a kinetic isotope effect from deuterium at C-4 and should be slower in deuterium oxide than in water due to the relative weakness of the catalyzing acid in the former solvent.[10]

The requisite deuterated substrate was prepared by heating 17β-hydroxyandrost-5-en-3-one with deuterium oxide in diglyme (Figure 3). Deuterium exchange at C-4 was shown to be quite rapid, but since the 4,4-dideuterated compound was desired, the heating period was extended to 3 days. The product contained 1.84 atoms of deuterium and although it was a mixture, the 4,4-dideuterated species was the principal component (D_0, 7.2%; D_1, 12.7%; D_2, 68.6%; and D_3, 11.5%). The kinetics of isomerization in 0.12 *N* hydrochloric acid–water were studied spectrophotometrically *vs.* the nondeuterated standard and for the pseudo-first-order reaction gave k_H/k_D = 4.1 (see Table I and Figure 2). Due to the small amount of non- and monodeuterated species this is a minimal figure for the isotope effect and clearly establishes a rate-determining loss of a C-4 proton in accord with an enolization mechanism. The nondeuterated Δ^5-3-ketone was also allowed to isomerize with 0.12 *N* deuteriohydrochloric acid–deuterium oxide (88%) and found to undergo reaction at a faster rate than in water, k_{D_2O}/k_{H_2O} = 1.56. Since there was 12% of protiated water and acid present the true k_{D_2O}/k_{H_2O} was actually 1.64, which again is consistent with only the enolization mechanism A. This establishes conclusively that isomerization of the β,γ-unsaturated ketone proceeds *via* enolization and that the neutral enol, once it has been formed, undergoes preferential protonation at the γ-position (C-6) in contrast to the enolate anion which undergoes preferential protonation at the α-position (C-4). Further, it is apparent from the deuterium incorporation studies that there is but little dissociation of the enol in acid medium. Although this would seem to establish that C-protonation of the enolate anion is faster than O-protonation, studies in progress indicate that formation of the β,γ-unsaturated ketone by acid

(9) R. C. Cookson, D. P. G. Hamon, and R. E. Parker, *J. Chem. Soc.*, 5014 (1962).

(10) See F. A. Long, *Ann. N. Y. Acad. Sci.*, **84**, 596 (1960).

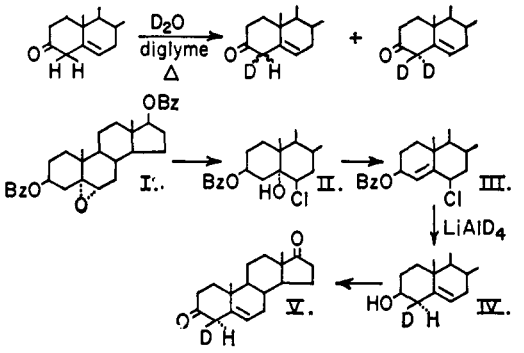


Figure 3.

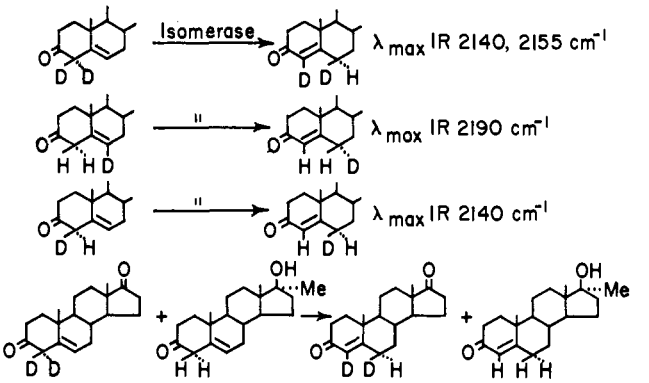
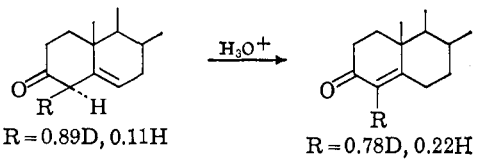


Figure 4.

quenching of the enolate anion may be due to high local concentrations of base and that O-protonation is in fact the fastest step. The question of C- *vs.* O-protonation, while of fundamental interest, has no bearing on the mechanism of the enzymatic process and will be treated in a subsequent paper.

It was now of interest to establish the stereochemistry of proton loss from C-4 in the enolization process. For this purpose and for the enzymatic studies a stereospecifically C-4 deuterated Δ^5-3-ketone was required. It has been shown[11] that lithium aluminum deuteride reduction of 3-benzoyloxy-6β-chlorocholest-4-ene yields 4β-deuterio-3β-hydroxycholest-5-ene. Adaptation of this synthesis to the requisite C_{19} series proceeded *via* 3β,17β-dihydroxy-5α,6α-oxidoandrostane dibenzoate (I), (Figure 3). Cleavage of the epoxide with hydrochloric acid gave the 5,6-chlorohydrin (II) which was dehydrated with thionyl chloride–pyridine to provide the dibenzoate of 6β-chloro-3β,17β-dihydroxyandrost-4-ene (III). Lithium aluminum deuteride reduction gave 4β-deuterio-3β,17β-dihydroxyandrost-5-ene (IV), which was oxidized by Jones[12] reagent to the desired 4β-deuterioandrost-5-ene-3,17-dione (V). Due to the extremely facile exchange of C-4 hydrogen or deuterium in the β,γ-unsaturated ketone, which occurred on occasion when the sample was simply recrystallized, deuterium analysis of V was carried out by hydride reduction to the Δ^5-diol (IV). While the diol IV contained 1.0 atom of deuterium before Jones oxidation, oxidation followed by reduction and analysis indicated the loss of 0.11 atoms which presumably occurred during the oxidation step. For all kinetic runs, a sample of IV containing 1.0 atom of deuterium was freshly oxidized to the 4β-deuterio ketone (V) which was assumed to contain 0.89 atom of deuterium in all cases.

The rate of acid-catalyzed isomerization for this 4β-deuterio compound was studied as described for the dideuterated compound and gave $k_H/k_D = 1.51$. The material was also isomerized to completion on a preparative scale and the α,β-unsaturated ketone which was formed contained 0.78 atom of deuterium remaining at C-4. From these data it is possible to calculate the relative rate of 4β- and 4α-proton loss as well as the true 4β-deuterium isotope effect; $k_{4\beta H}/k_{4\alpha H} = 0.86$ and $k_{4\beta H}/k_{4\beta D} = 6.1$. The calculated isotope effect is dependent upon a precise knowledge of the deuterium content of the starting β,γ-unsaturated ketone, however, and if, for example, V contained 0.91 instead of 0.89 atom, the respective values would be $k_{4\beta H}/k_{4\alpha H} = 0.85$ and $k_{4\beta H}/k_{4\beta D} = 5.1$. Nevertheless it is clear that the 4β-isotope effect is substantial and that the equatorial 4α-proton is lost somewhat more rapidly than the 4β-axial proton. The fact that there was no great preference for axial proton loss demonstrates that stereoelectronic[13] control is not important in this case and is in accord with a transition state bearing considerable enolic character where proton loss might be expected to occur from the least hindered α-face of the steroid. However, it should be noted that with respect to the C-6 position proton loss and gain with strong acid are favored from the β-axial position despite the fact that the transition state bears considerable enolic character.[6]



Enzymic Isomerization

Stereochemistry. The finding of Talalay and coworkers[4,5] that minimal quantities of deuterium were incorporated during enzymic isomerization carried out in deuterium oxide indicated that the net process involved a transfer of a proton from the C-4 position of the β,γ-unsaturated ketone to the C-6 position of the α,β-unsaturated ketone. The net intramolecular transfer of hydrogen was readily established by the isomerization of several C-4 deuterated substrates (Figure 4). 17β-Hydroxyandrost-5-en-3-one which had been partially deuterated at C-4 (1.19 atoms of D) was enzymatically converted to the α,β-unsaturated ketone (testosterone) which contained 1.07 atoms of deuterium. The infrared spectrum showed strong bands in the C–D stretching region at 2255 and 2140 cm.$^{-1}$ due to deuterium at C-4 and at C-6β, respectively. Similarly, 17β-hydroxy-17α-methylandrost-5-en-3-one containing 0.71 atom of deuterium at C-4 gave 17α-methyltestosterone containing 0.57 atom of deuterium at C-4 and at the axial 6β-position. This established the net deuterium transfer process as well as the stereospecific nature of the transfer to the 6β-position. Further

(11) R. E. Ireland, T. I. Wrigley, and W. G. Young, *J. Am. Chem. Soc.*, **81**, 2818 (1959).
(12) A. Bowers, T. G. Halsall, E. R. H. Jones, and A. J. Lemin, *ibid.*, **75**, 2548 (1953).
(13) E. J. Corey and R. A. Sneen, *ibid.*, **78**, 6269 (1956).

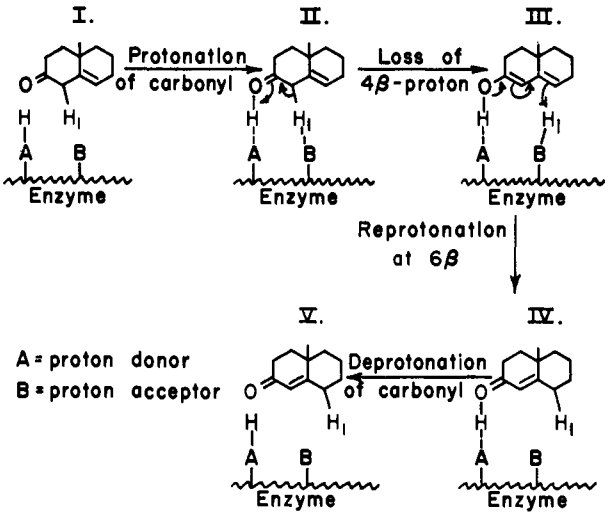


Figure 5. Proposed mechanism of isomerase action.

confirmation of the stereochemistry came from the enzymatic isomerization of 17β-hydroxyandrost-5-en-3-one containing 0.2 atom of deuterium on the double bond at C-6. In this case the resulting testosterone exhibited only a single deuterium band at 2190 cm.$^{-1}$, establishing a proton transfer to the 6β-position and the presence of deuterium at the 6α-position. The acid-catalyzed isomerization of the same starting material also gave 6α-deuterated testosterone.

To establish the stereochemistry of transfer from C-4, the 4β-monodeuterated androst-5-ene-3,17-dione containing 0.89 atom of deuterium was isomerized to yield androst-4-ene-3,17-dione containing 0.6 atom of deuterium at the 6β-position. No deuterium could be detected at C-4 by infrared or by n.m.r. analysis and *therefore the net enzymatic process involved an axial–axial transfer from C-4β to C-6β.*

Intra- vs. Intermolecular Proton Transfer. This did not, however, establish yet whether the net transfer was of an intra- or intermolecular nature. In fact, an ingenious scheme has been proposed[14] whereby a group on the enzyme such as RNH_2 could protonate the double bond at C-6 and remove a proton from C-4 in a cyclic concerted process. The proton removed from C-4 would then be passed on to the next or to a subsequent molecule of β,γ-unsaturated ketone reacting with the enzyme.

To eliminate the possibility of such an intermolecular transfer, two substrates, 4,4-dideuterated androst-5-ene-3,17-dione and 17α-methyl-17β-hydroxyandrost-5-en-3-one were simultaneously isomerized in equal concentrations and the α,β-unsaturated ketone products separated and analyzed by mass spectroscopy and infrared spectroscopy. No deuterium could be detected in the 17-methyl derivative, and when the cross-over experiment was run with deuterated 17-methyl compound and nondeuterated 17-keto compound no deuterium could be detected in the latter (Figure 4). While such an experiment invariably suffers the risk of dissimilar rates and/or enzyme binding constants, the individual rates of isomerization of the dideuterated 17-keto and nondeuterated 17-methyl substance were of the same order of magnitude. Additional evidence against intermolecular deuterium transfer came from the isomerization of a mixture of non-, mono-, and dideuterated derivatives of a single substrate. A significant degree of intermolecular transfer would involve a transfer of deuterium from dideuterated to nondeuterated species and would therefore lead to an increase of monodeuterated and decrease of non- and dideuterated species in the product. While such analysis was complicated by small net losses of deuterium in several of the experiments, no evidence could be found that deuterium was transferred to the nondeuterated compound and *it may be concluded that the isomerization involves intramolecular proton transfer.*

4β-Deuterium Isotope Effect. Determination of the magnitude of the 4β-deuterium isotope effect was highly pertinent in establishing the mechanism of the enzymatic reaction. Velocity–concentration curves of androst-5-ene-3,17-dione and of its 4β-deuterio analog containing 0.89 atom of deuterium established a highly significant isotope effect. Reciprocal Lineweaver–Burk[15] plots gave Michaelis constants of 3.1×10^{-4} and 1.4×10^{-4} M for the nondeuterated and deuterated compound, respectively, and, at maximal velocity, $V_{\max H}/V_{\max D} = 5.35$.

Mechanism. The intramolecular nature of the enzymatic proton transfer and the high deuterium isotope effect clearly point to an enolic type isomerization mechanism as has already been suggested by Talalay and co-workers[5] on the basis of difference spectra studies. The mechanism which appears to be most consistent with our data is shown in Figure 5 and is closely related to the chemical process. Assume a proton donor group (AH) on the enzyme and a proton acceptor (B). Coordination of AH with the steroid carbonyl by hydrogen bonding or full hydrogen transfer, concomitant with or followed by abstraction of the C-4β proton by B, yields the enzyme–enol intermediate III in a low energy push–pull type mechanism. Protonation at the 6β-position by BH, which can occur with a minimum of bond stretching due to the diaxial nature of the transfer, yields the protonated α,β-unsaturated ketone. Removal of the carbonyl proton then gives the α,β-unsaturated ketone and regenerates the enzyme. The high isotope effect which accompanies the introduction of a deuterium atom at the 4β-position can theoretically arise either in the enolization step or in the reprotonation step since deuterium is transferred in both steps. In common with the chemical mechanism and on the assumption that the enzyme–enol intermediate will be the highest energy complex present, we favor loss of the C-4 proton as the rate-determining step. The assumption that it is the enol rather than the enolate anion which undergoes protonation stems from the strictly chemical finding that C-protonation of the anion is favored at C-4 rather than C-6.[7,8] The requirement that BH or BD does not exchange with protons or deuterium of the medium is readily explicable on the basis of a very fast reprotonation step and the probability that in the enzyme–enol complex BH is shielded from solvent. It might in fact be suggested that the extremely fast rate of this enolization and reprotonation depends upon reaction occurring in a hydrophobic region. The small deuterium losses

(14) W. R. Nes, E. Loeser, R. Kirdani, and J. Marsh, *Tetrahedron*, **19**, 299 (1963).

(15) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

occurring in the isomerization of C-4 deuterated substrates may be due to exchange of BD with the water of the medium or to dissociation of the enzyme–enol complex (III) and protonation of the enol by water. As in the case of the closely related phosphoglucose-isomerase[16] reaction, this deuterium loss was shown to be temperature dependent since androst-5-ene-3,17-dione containing 0.78 atom of deuterium at C-4 lost only 0.01 atom of isotope when isomerization was carried out at 15°, but 0.05 atom was lost at 22°.

The nature of the postulated proton donor (AH) and proton acceptor (B) can only remain speculative at the present time. On the basis of the pH profile[5] which is essentially flat from pH 6–9, AH could be a phenol or NH group while B could be an amino group or carboxylate anion. An attractive possibility[17] is the utilization of an imidazole molecule which by simple bond flicking could act both as proton donor and acceptor. Talalay has presented evidence in favor of this hypothesis by demonstrating that an intact histidine residue is necessary for isomerase activity.

Kinetics. From a kinetic point of view the isomerase reaction can be pictured by the following equation.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} E\text{–}S \underset{k_4}{\overset{k_3}{\rightleftarrows}} E\text{–}S' \underset{k_6}{\overset{k_5}{\rightleftarrows}} E\text{–}P \underset{k_8}{\overset{k_7}{\rightleftarrows}} E\text{–}P$$

S = Δ^5-3-ketone
S′ = enol
P = Δ^4-3-ketone

Since the over-all equilibrium of the reaction lies far to the right and since the Δ^4-3-ketone product is not an inhibitor of the reaction, the reverse steps k_8 and k_6 can be ignored. The high magnitude of the over-all isotope effect indicates that k_4 is small compared to k_5. The reverse situation would lead to a relative increase in rate for the deuterio compound since k_4 would be subject to an isotope effect. Also, from the chemical arguments already developed, it is unlikely that reprotonation of the enol occurs at C-4 as readily as at C-6. On this basis the over-all equation can then be reduced to the classical Michaelis–Menten equation $E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} E\text{–}S \xrightarrow{k_3} E + P$, where k_3, the slow step of the reaction, is now the rate of enolization and equivalent to the turnover number. Under these conditions, the Michaelis constant $K_m = (k_2 + k_3)k_1$ and if $k_3 >> k_2$, $K_m = k_3/k_1$ or if $k_2 >> k_3$, $K_m = k_2/k_1$ (see Table II).

Table II. K_m and Relative V_{max} Values for the Isomerization of Androst-5-ene-3,17-dione and its 4β-Deuterio Derivative

Substrate	K_m[a]	V_{max}(rel.)	k_H/k_D
Non-D	3.1×10^{-4}	100[b]	5.35
4β-D[c]	1.4×10^{-4}	18.7	

[a] Wang, *et al.*, ref. 5, report $K_m = 3.2 \times 10^{-4}$ for non-D. [b] $V_{maxH} = 17 \times 10^6$ min. (ref. 5). [c] 0.89 atom of deuterium.

If the assumption is made that k_1 and k_2 are essentially identical for the deuterated and nondeuterated compounds and that the higher K_m of the nondeuterated substance is strictly a reflection of the faster rate of enolization (k_3), the constants k_1 and k_2 can be calculated for each substrate. This leads to the following. Under these conditions the true enzyme–substrate equilibrium constant is equal to 1.0×10^{-4} and k_2 and k_3 are both of the same order of magnitude.

$$E + S \underset{k_2 = 8.2 \times 10^6 \text{ min.}^{-1}}{\overset{k_1 = 8.1 \times 10^{10}\ M^{-1} \text{ min.}^{-1}}{\rightleftarrows}} E\text{–}S \xrightarrow[k_{3D} = 3.2 \times 10^6 \text{ min.}^{-1}]{k_{3H} = 17 \times 10^6 \text{ min.}^{-1}} E + P$$

Experimental[18]

Deuterioacetic Acid Catalyzed Hydrolysis of 3-Ethoxycholesta-3,5-diene. A suspension of 100 mg. of 3-ethoxycholesta-3,5-diene in 1 ml. of 50% deuterioacetic acid–deuterium oxide and 0.5 ml. of diglyme was warmed on the steam bath for 10 min. and then stirred for 1 hr. at room temperature. Water was added and pure cholest-4-en-3-one, 85 mg., m.p. 81–82°, was obtained by ether extraction followed by thin layer chromatography on silica gel. In the infrared the principal C–D stretching band appeared at 2140 cm.$^{-1}$ (6β-D), whereas the 2255-cm.$^{-1}$ band (4D-Δ^4) was completely absent. The out-of-plane deformation band for the C-4 proton had shifted from 869 to 854 cm.$^{-1}$. In the n.m.r. spectrum the C-4 proton appeared as a sharp peak at 345 c.p.s. (half-line width 2.4 c.p.s.[19] Found[20]: 1.0 atom of deuterium).

Deuterium Chloride Catalyzed Hydrolysis of 3-Ethoxycholesta-3,5-diene. A suspension of 50 mg. of 3-ethoxycholesta-3,5-diene and 0.5 ml. of 0.5 *N* deuterium chloride–deuterium oxide (99%) in 5 ml. of dry diglyme was warmed on the steam bath to a clear solution and then allowed to stand for 5 min. without further heating. Dilution with deuterium oxide resulted in the separation of a yellow precipitate which was shown (by thin layer, silica gel chromatography) to be a 9:1 mixture of cholest-4-en-3-one and the starting material. In the infrared, the pure Δ^4-3-ketone exhibited a strong band at 2140 (6β-D) with no band visible at 2255 cm.$^{-1}$ (4D-Δ^4). In the n.m.r. the C-4 proton appeared as a sharp peak at 345 c.p.s. (half-line width 2.4 c.p.s. Found[20]: 0.9 atom of deuterium).

Deuterium Chloride Catalyzed Isomerization of Cholest-5-en-3-one. A solution of 50 mg. of cholest-5-en-3-one and 0.5 ml. of 0.5 *N* deuterium chloride–deuterium oxide solution in 5 ml. of dry diglyme was stirred for 5 min. at room temperature followed by dilution with an excess of deuterium oxide. The resulting precipitate was filtered and the pure Δ^4-3-one (40 mg.) was separated by thin layer, silica gel chromatography. The principal C–D stretching band in the infrared appeared at 2140 (6β-D), while a very weak band at 2255 cm.$^{-1}$ demonstrated the presence of a

(16) I. A. Rose and E. L. O'Connell, *J. Biol. Chem.*, **236**, 3086 (1961).
(17) Presented by P. Talalay, 2nd International Congress of Endocrinology, London, Aug. 1964.
(18) All infrared analyses were carried out in chloroform solution in a Beckman IR-7 with Bausch and Lomb replica grating; n.m.r. spectra were obtained with a Varian 4300 spectrometer at a frequency of 60 Mc./sec. The samples were dissolved in deuteriochloroform and spectra were calibrated using the side-band technique. Peak positions are reported in cycles per second downfield from tetramethylsilane (internal reference). We are grateful to Mr. N. Bacon and George Scrimshaw for the infrared and to Mr. F. Cronin and T. A. Wittstruck for the n.m.r. determinations. The kinetic enzyme runs were carried out with the able technical assistance of Mr. H. Lawrence, Jr.
(19) T. A. Wittstruck, S. K. Malhotra, and H. J. Ringold, *J. Am. Chem. Soc.*, **85**, 1699 (1963).
(20) Deuterium analysis by Mr. Josef Nemeth, Urbana, Ill.

minor amount of 4D-Δ^4 species (Found: 1.06 atoms of deuterium).

In another experiment the reaction was terminated after 70% isomerization and the unreacted Δ^5-3-one, which was separated by thin layer, silica gel chromatography, failed to exhibit a C–D stretching band in the infrared.

Deuterium Chloride Catalyzed Isomerization of 17β-Hydroxyandrost-5-en-3-one, Stopped Short of Completion. A solution of 300 mg. of the Δ^5-3-ketone and 1 ml. of 0.5 *N* deuterium chloride–deuterium oxide in 10 ml. of diglyme was stirred for 3 min. at room temperature. The product, which was isolated by the addition of water, was shown to have undergone approximately 50% isomerization based on thin layer chromatography and integration of the carbonyl absorption bands at 5.85 (Δ^5-3-one) and 6.0 μ (Δ^4-3-one). Without purification, the mixture was reduced with lithium aluminum hydride in anhydrous ether and from the resulting mixture of diols, pure 3β,17β-dihydroxyandrost-5-ene, 100 mg., m.p. 175–178°, was separated by silica gel column chromatography (ethyl acetate–benzene, 1:3) and analyzed for deuterium content (Found[20]: 0.08 atoms of deuterium).

Deuterium Chloride Catalyzed Enolization of Cholest-4-en-3-one. A solution of 50 mg. of cholest-4-en-3-one and 0.5 ml. of 0.5 *N* deuterium chloride–deuterium oxide in 5 ml. of dry diglyme was stirred for 7 hr. at room temperature followed by dilution with an excess of deuterium oxide. The precipitate was filtered, washed, and dried. Recrystallization from acetone–hexane gave 40 mg. of pure Δ^4-3-one which, in the infrared, exhibited a principal C–D stretching band at 2140 (2β- and 6β-D), a weak band at 2220, and a very weak band at 2255 cm.$^{-1}$ (4D-Δ^4), while the C-4 out-of-plane deformation band had shifted from 869 to 854 cm.$^{-1}$. In the n.m.r. the C-4 proton appeared as a sharp singlet at 345 c.p.s. (half-line width 2.4 c.p.s.; integrated area 0.9 proton. Found[20]: 1.5 atoms of deuterium).

In another experiment terminated after 24 hr. the total deuterium incorporation was 2.4 atoms with 0.2 atoms at C-4.

Preparation of 5α,6α-Oxido-3β,17β-dihydroxyandrostene Dibenzoate. A solution of 2 g. of 3β,17β-dihydroxyandrost-5-ene dibenzoate and 1.7 g. of 80% *m*-chloroperbenzoic acid in 50 ml. of methylene chloride was left overnight at room temperature. The mixture was washed with aqueous sodium bicarbonate and water and then dried and concentrated *in vacuo* to give 1.9 g. of a crystalline solid, m.p. 235–240°. Recrystallization from acetone gave 1.5 g. of pure epoxide, m.p. 239–241°. In the n.m.r. the 6β-proton appeared as a doublet at 176 and 179.5 c.p.s., which is consistent only with coupling between the 6β- and 7β-protons and hence the α-oxido structure.

Anal. Calcd. for $C_{33}H_{38}O_5$: C, 77.01; H, 7.44. Found: C, 76.87; H, 7.29.

Preparation of 6β-Chloro-3β,5α,17β-trihydroxyandrostane 3,17-Dibenzoate. To a solution of 1.4 g. of the α-oxide in 70 ml. of acetone, 1.2 ml. of concentrated hydrochloric acid (37%) was added. The solution was allowed to stand for 30 min. at room temperature and then slowly diluted with water to yield 1.4 g. of the chlorohydrin, m.p. 223–225°. Recrystallization from acetone–water gave an analytical specimen of m.p. 225–228°.

Anal. Calcd. for $C_{33}H_{39}ClO_5$: C, 71.97; H, 7.14. Found: C, 71.64; H, 7.37.

Preparation of 6β-Chloro-3β,17β-dihydroxyandrost-4-ene Dibenzoate. To an ice-cooled solution of 2 g. of the chlorohydrin in 20 ml. of dry pyridine, 2 ml. of thionyl chloride was added slowly. After standing for 20 min. at 0°, the yellow solution was poured onto crushed ice and the product (1.8 g., m.p. 164–167°) was filtered, washed, and dried. Recrystallization from acetone–hexane afforded pure material of m.p. 167–169°. In the n.m.r., the 19-methyl group was shifted downfield to 83.5 c.p.s. due to magnetic deshielding by the 6β-chloro group, and the 6α-proton appeared as a triplet centered at 278 c.p.s. ($J_{6\alpha H,7\beta H}$ = 2.5 c.p.s., $J_{6\alpha H,7\alpha H}$ = 2.5 c.p.s.).

Anal. Calcd. for $C_{33}H_{37}ClO_4$: C, 74.40; H, 7.00. Found: C, 74.65; H, 7.27.

Preparation of 4β-Deuterio-3β,17β-dihydroxyandrost-5-ene. A mixture of 1.5 g. of 6β-chloro-3β,17β-dihydroxyandrost-4-ene dibenzoate and 0.5 g. of lithium aluminum deuteride in 75 ml. of anhydrous ether was boiled under reflux for 2 hr. and then stirred overnight at room temperature. The excess hydride was decomposed by the addition of a saturated solution of sodium sulfate and the product was isolated by extraction with ethyl acetate. Chromatography on silica gel (40 g.) gave the desired diol (0.7 g.) in the benzene–ethyl acetate (1:4) eluate. One recrystallization from acetone–hexane gave material of m.p. 175–178° which exhibited the same polarity as the non-deuterated substance on thin layer chromatography and gave no melting point depression on admixture. In the infrared the 4β-deuterio C–D stretching band appeared at 2140 cm.$^{-1}$. Analysis by mass spectrum demonstrated the presence of 1.0 atom of deuterium (Found[21]: D_0, 0%; D_1, 100%).

Preparation of 4β-Deuterioandrost-5-ene-3,17-dione. To an ice-cooled solution of 100 mg. of the 4β-deuterio diol in 10 ml. of acetone, 0.5 ml. of 8 *N* Jones reagent[12] was added with stirring. After stirring for another 3 min. the resulting turbid solution was poured through a cotton plug into a mixture of crushed ice and salt. Water was added and the crystalline solid was rapidly filtered, washed, and dried. Recrystallization from aqueous acetone provided 60 mg. of 4β-deuterio-Δ^5-dione, m.p. 140–143°,[22] which, in the infrared and ultraviolet, exhibited only traces of conjugated Δ^4-3-ketone. The 4β-deuterio C–D stretching band appeared in the infrared at 2125 cm.$^{-1}$. In order to determine the deuterium content of the dione, it was reduced with lithium aluminum hydride to the diol, which was then analysed by mass spectrum (Found[21]: 0.89 atom of deuterium (D_0, 11% and D_1, 89%)).

General Procedure for the Preparation of 4,4-Dideuterated Δ^5-3-Keto Steroids. A solution of 10 mmoles of

(21) We are grateful to Dr. R. Ryhage, Karolinska Institutet, Stockholm, and Professor K. Biemann, Massachusetts Institute of Technology, Cambridge, Mass., for the mass spectral analyses.

(22) For the nondeuterated substance, C. Djerassi, R. R. Engle, and A. Bowers, *J. Org. Chem.*, **21**, 1547 (1956), and W. R. Nes, *et al.*, ref. 14, report m.p. 119–125°, while A. Butenandt and J. Schmidt-Thome, *Ber.*, **69**, 882 (1936), and K. E. Pfitzner and J. G. Moffat, *J. Am. Chem. Soc.*, **85**, 3028 (1963), report m.p. 158° and 167–169°, respectively.

Δ^5-3-keto steroid in 35 ml. of dry diglyme and 6 ml. of deuterium oxide was heated at reflux for 15 min. Water was added and the precipitate was filtered and dried. The material, recovered in almost quantitative yield, was shown to be unisomerized by infrared and thin layer analysis. The principal C–D stretching bands appeared in the infrared at 2125 (4βD) and 2220 cm.$^{-1}$ (4α-D or 4,4-diD). The deuterium content[20] in representative runs was as follows for three substrates: 17β-hydroxyandrost-5-en-3-one, 1.55 atoms; androst-5-ene-3,17-dione, 0.70 atom; and 17α-methyl-17β-hydroxyandrost-5-en-3-one, 0.77 atom.

Preparation of 4,4-Dideuterioandrost-5-ene-3,17-dione. A solution of 1 g. of 17β-hydroxyandrost-5-en-3-one in 10 ml. of dry diglyme and 2 ml. of deuterium oxide was boiled for 3 days. The product, after isolation by the usual procedure, was reduced with 500 mg. of lithium aluminum hydride in anhydrous ether. The pure Δ^5-diol, m.p. 175–178°, was separated by column chromatography (40 g. of silica gel) on elution with 20% benzene–ethyl acetate. In the infrared a C–D stretching band appeared at 2205 cm.$^{-1}$. The compound was analyzed by mass spectrum (Found[21]: 1.84 atoms of deuterium (D_0, 7.2%; D_1, 12.7%; D_2, 68.6%; and D_3, 11.5%)).

This diol was oxidized by Jones reagent to the 4,4-dideuterated androst-5-ene-3,17-dione which was used for the determination of k_H/k_D in the acid-catalyzed isomerization of the Δ^5-3-one.

Preparation of 6-Deuterated-17β-hydroxyandrost-5-en-3-one. A solution of 290 mg. of 6β-deuteriotestosterone (D = 0.7 atom) and 1.1 g. of potassium *t*-butoxide in 5 ml. of anhydrous *t*-butyl alcohol was stirred at room temperature, under nitrogen for 2 hr. Cold 10% acetic acid (25 ml.) was added, followed by further dilution with water. The precipitate was filtered, washed, dried, and recrystallized from acetone and water, yielding 200 mg. of β,γ-unsaturated ketone, m.p. 160–163°. In the infrared the C-6 deuterium on a double bond appeared at 2235 cm.$^{-1}$ (Found[21]: 0.22 atom of deuterium).

Hydrochloric Acid Catalyzed Isomerization of 4β-Deuterioandrost-5-ene-3,17-dione. To 500 ml. of 0.12 *N* hydrochloric acid solution (0.1 *M* in lithium perchlorate) a solution of 10 mg. of 4β-deuterioandrost-5-ene-3,17-dione (0.89 atom of deuterium) in 12 ml. of anhydrous methanol was added. The solution was stirred for 40 min. at 27° and the steroid was isolated by extraction with ethyl acetate. Isomerization to the Δ^4-3-one was shown to be complete by thin layer and infrared analysis. Recrystallization from acetone–hexane gave 4 mg. of androst-4-ene-3,17-dione, m.p. 172–174°, infrared maximum 2255 cm.$^{-1}$ (Δ^4-4D) (Found[21]: 0.78 atom of deuterium (D_0, 22% and D_1, 78%)).

Isomerization in diglyme as solvent was carried out in the following manner. A solution of 6 mg. of the 4β-deuterio-Δ^5-dione and 0.5 ml. of 1 *N* hydrochloric acid in 5 ml. of dry diglyme was stirred for 40 min. at room temperature. Water was added and the steroid was isolated by extraction with ether. Recrystallization from acetone–hexane gave the Δ^4-dione which contained 0.72 atom of deuterium at C-4 (D_0, 28% and D_1, 72%).

Hydrochloric Acid Catalyzed Isomerization of 6-Deuterated 17β-Hydroxyandrost-5-en-3-one. Isomerization of the material containing 0.22 atom of deuterium was carried out in diglyme by the procedure described above. The testosterone which was formed exhibited a C–D stretching band at 2190 cm.$^{-1}$ (6α-D) and was found by mass spectrum to contain 0.19 atom of deuterium.

Kinetics of Isomerization. A. Acid Catalyzed. The rates of isomerization of androst-5-ene-3,17-dione, of its 4β-deuterio derivative containing 0.89 atom of deuterium, and of its 4,4-dideuterated derivative containing 1.84 atoms of deuterium (D_0, 7.2%; D_1, 12.7%; D_2, 68.6%; and D_3, 11.5%) were determined by the following procedure.

A solution of 7.5 mg. of steroid in 5 ml. of methanol was prepared, and 0.05 ml. of this solution was added to 2 ml. of 0.12 *N* hydrochloric acid (0.1 *M* in lithium perchlorate) contained in a 3-ml. cuvette with a light path of 1 cm. The optical density of the resulting solution was measured at 250 mμ at different time intervals with a Cary Model 11 ultraviolet spectrophotometer, the blank being the same volume of hydrochloric acid and methanol. The temperature of the solution was maintained at 27° throughout the reaction. The rate of isomerization was measured by the increase in optical density due to formation of the α,β-unsaturated ketone and the reaction was followed until no further change occurred. The difference between the final optical density reading $(O.D.)_\infty$ and the reading at a given time $(O.D.)_t$ represented the amount of unisomerized Δ^5-3-ketone remaining at that time. A plot of $\log[(O.D.)_\infty - (O.D.)_t]$ *vs.* time gave a straight line. In the case of the deuterated analogs, some deviation from straight line behavior was observed in the latter part of the reaction, very likely due to isotope enrichment in the unisomerized material. Therefore, points from only the first half-life of reaction were utilized to calculate the pseudo-first-order rate constants which are listed in Table I (k = 2.303 × slope). The rate of isomerization of androst-5-ene-3,17-dione in *ca.* 88% deuterium oxide was determined by adding 75 μg. of steroid in 0.05 ml. of methanol to 2 ml. of 0.12 *N* acid solution which was prepared by diluting 3 ml. of 1 *N* hydrochloric acid to 25 ml. with 99.8% deuterium oxide. From the loss of deuterium (0.11 atom) in the preparative isomerization of the 4β-deuterio-Δ^5-3-keto compound which initially contained 0.89 atom of deuterium and from the apparent kinetic isotope effect ($k_H/k_{4\beta D}$ = 1.51) the true 4β-deuterium isotope effect and the relative rate of 4β- *vs.* 4α-proton loss were calculated in the following manner. Let x, y, and z equal the relative rate of loss of the 4β-proton, the 4α-proton, and the 4β-deuteron, respectively. Since the kinetic isotope effect was 1.51, $11x + 89z + 100y = 100/1.51 = 66.3$. Of the 89 atom % of starting material that contained deuterium, 11 atom % of 4β-deuterium and 78 atom % of 4α-proton reacted; therefore, $y = 78/11z$. Finally, since $x + y = 1$, the three relative rates can be solved: 4α-H = 1, 4β-H = 0.86, 4β-D = 0.077, and the true $k_{4\beta H}/k_{4\beta D}$ = 6.1.

B. Enzyme Catalyzed. The rates of isomerization of androst-5-ene-3,17-dione and the corresponding 4β-deuterio derivative containing 0.89 atom of deuterium

were determined as follows. Twice-recrystallized isomerase[23] of specific activity 169,000 units/mg. was dissolved in 0.03 *M* phosphate buffer, pH 7.0, at a concentration of 0.1 μg./ml. The steroid (six concentrations from 12.5 to 74 μg.) was added in 0.05 ml. of ethanol to 2.4 ml. of phosphate buffer contained in a 3.0 ml. cuvette and the reaction was initiated immediately thereafter by the addition of 0.025 to 0.050 ml. of enzyme solution. All runs were made at 27° utilizing a Cary Model 11 recording spectrophotometer and zero-order rates were determined for the initial 20 sec. of reaction. Maximal velocity and K_m for each substrate were determined by double-reciprocal plots of concentration and velocity by the method of Lineweaver and Burk. Lines were fitted to the experimentally determined points by the method of least squares. The average values for five runs are shown in Table II and are uncorrected for the 0.11 atom of hydrogen present in the deuterated substrate.

Preparative Enzymatic Isomerization of Deuterated Substrates. All reactions were carried out at room temperature by the addition of a freshly prepared solution of 7.5 mg. of Δ^5-3-keto steroid in 5 ml. of 95% ethanol to 250 ml. of phosphate buffer (pH 7.0, 0.03 *M*). A solution of 10 μg. of isomerase (specific activity 169,000 units/mg.) in 0.5 ml. of the same buffer was added to the stirred steroid solution, and the reaction was allowed to proceed for 5 min. before the product was isolated by extraction with ethyl acetate. Purification of the Δ^4-3-ketone was effected by thin layer chromatography on silica gel and deuterium content was determined by mass spectrometry.

4,4-Dideuterated 17β-Hydroxyandrost-5-en-3-one. The Δ^5-3-ketone analyzing for 1.19 atoms of deuterium[21] (D_0, 24.1%; D_1, 34.3; D_2, 40.1%; D_3, 1.4%; and D_4, 0.2%) gave 5.0 mg. of testosterone, m.p. 155–157°, containing 1.09 atoms of deuterium (D_0, 24.3%; D_1, 46.5%; D_2, 29.0%; D_3, 1.3%; and D_4, 0.3%). Carbon–deuterium stretching bands appeared at 2140 (6β-D) and 2255 cm.$^{-1}$ (Δ^4-4-D) in the infrared.

4,4-Dideuterated 17β-Hydroxy-17α-methylandrost-5-en-3-one. Isomerization of material containing 0.71 atom of deuterium[20] gave 4.5 mg. of 17α-methyltestosterone, m.p. 162–164°, containing 0.57 atom of deuterium. Characteristic bands for Δ^4-4-deuterium and 6β-deuterium were found at 2255 and 2140 cm.$^{-1}$.

6-Deuterated 17β-Hydroxyandrost-5-en-3-one. The Δ^5-3-ketone containing 0.22 atom of deuterium gave 6α-deuterated testosterone with the same deuterium content. The only deuterium band visible in the infrared appeared at 2190 cm.$^{-1}$.

4β-Deuterioandrost-5-ene-3,17-dione. The sterically homogeneous 4β-deuterio substance containing 0.89 atom of deuterium gave 4.5 mg. of 6β-deuterioandrost-4-ene-3,17-dione, m.p. 172–174°, containing 0.6 atom of deuterium (D_0, 40% and D_1, 60%). Only a single deuterium band appeared (2140 cm.$^{-1}$, 6β-D) in the infrared.

1:1 Mixture of 4,4-Dideuterated Androst-5-ene-3,17-dione and Nondeuterated 17β-Hydroxy-17α-methylandrost-5-en-3-one. A mixture of 7.5 mg. of each substrate was isomerized as described above but with double the volume of ethanol, buffer, and enzyme solution. Separation by thin layer chromatography demonstrated that isomerization was complete and provided each Δ^4-3-ketone in a pure state. The starting deuterated substance contained 0.80 atom of deuterium[20] and the androst-4-ene-3,17-dione product contained 0.77 atom (D_0, 34.8%; D_1, 53.0%; and D_2, 12.2%). The 17α-methyltestosterone was shown to be free of deuterium both by mass analysis and infrared.

1:1 Mixture of Nondeuterated Androst-5-ene-3,17-dione and 4,4-Dideuterated-17β-hydroxy-17α-methylandrost-5-en-3-one. The simultaneous isomerization of the mixed substrates by the procedure described above (deuterated Δ^5-3-ketone containing 0.77 atom of deuterium) gave 17α-methyltestosterone containing 0.74 atom of deuterium (D_0, 40.0%; D_1, 46.5%; and D_2, 13.5%) while the androst-4-ene-3,17-dione was found to be free of deuterium.

Influence of Temperature on Deuterium Loss in Enzymatic Isomerization. Duplicate isomerizations of 4,4-dideuterated androst-5-ene-3,17-dione were carried out by the usual procedure at 15 and 22°: starting material, 0.78 atom of D (D_0, 35.6%; D_1, 50.8%; and D_2, 13.6%); product at 15°, 0.77 atom of D (D_0, 36.4%; D_1, 49.5%; and D_2, 13.9%); product at 22°, 0.73 atom of D (D_0, 37.1%; D_1, 53.0%; and D_2, 9.9%).

(23) We are very grateful to Professor Paul Talalay for a generous gift of crystalline isomerase.