since small converted atomic motions can relax this energy without changing the interaction between subunits. It has been suggested before that the subunit interaction involves electrostatic energy in the salt bridges;[2] here we make a logical extension of this point and suggest that part of the energy between the subunit and its ligated heme can also be considered to be electrostatic. It is shown that binding of oxygen to the iron leads to significant transfer to positive charge to the π^* orbital of the porphyrin. The calculated stabilization of this positive charge by the permanent and induced protein dipoles increases by 1.1 kcal/mol upon the t → r transition. In particular, the r state stabilizes additional positive charge on the vinyl group and the C_b carbons of ring III, in agreement with substitution experiments. Thus we suggest that the electrostatic contribution to cooperativity in hemoglobin can be viewed as described schematically in Figure 6; the protein subunits are designed to have multiple folding conformations r and t, where the r state interacts more strongly than the t state with the charges of the heme-O_2 system while the t state interacts more strongly than the r state with the charges and dipoles of the neighboring subunits. The competition between intrasubunit electrostatic stabilization of the heme$^{\delta+}$-$O_2^{\delta-}$ system and intersubunit salt-bridge interactions is an important factor in cooperativity in hemoglobin.

Because of the appoximate nature of the electrostatics calculations, we cannot provide a quantitative estimate of the electrostatic contribution to $\Delta\bar{G}$ but wish rather to point out the feasibility of such an effect. A possibly interesting clue to the magnitude of this contribution might be provided by comparing the heme-heme interaction in bindings of CO and O_2 to hemoglobin; the cooperativity in CO binding is attributed to two factors: a steric barrier on the distal side[35] and protein strain at the proximal side. Since the strain contribution from the proximal side should be similar for CO and O_2, the additional cooperativity due to the distal effect should make $\Delta\bar{G}$ for CO binding larger[36] than for O_2 binding. Yet, $\Delta\bar{G}$ for CO is ~3.0 kcal/mol. We suggest that this is due to a smaller electrostatic contribution to cooperativity in HbCO than in HbO_2.

The present work might have general implications for other allosteric systems in showing that the allosteric effect may be associated with a balance of opposing electrostatic contributions. This is consistent with the idea that transfer of energy in biological systems is often associated with changes in electrostatic energy.[13]

Acknowledgment. This work was supported in part by Grant GM24492 from the National Institutes of Health and by the Alfred P. Sloan Foundation. We are grateful to Ms. Joyce Baldwin for providing us with the coordinates of HbCO.

(35) Szabo, A. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 2108-2111.

(36) The distal effect in CO is manifested in the "on rate", k_{on}, which is about 40 times larger in the R state than in the T state.[35] Assuming that the distal penetration barrier is determined by the steric resistance to a linear binding of CO and that the same steric strain still exists in the protein when the Fe-CO bond is completely formed, we would expect an extra distal contribution to $\Delta\bar{G}$.

A Reinvestigation of the Mechanism of *Pseudomonas testosteroni* Δ^5-3-Ketosteroid Isomerase

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Abstract: The mechanism of the isomerization of Δ^5-3-keto steroid by the enzyme of *P. testosteroni* has been reinvestigated with androst-5-ene-3,17-dione **1**. The formerly proposed 4β → 6β intramolecular transfer does not account for all the reaction. It has been demonstrated by using 4α- and 4β-deuterated substrates that this reaction is not stereospecific and involves a competitive abstraction of the 4α- and 4β-protons. The relative contribution of the two processes is temperature dependent. This may be attributed to some conformational change of the enzyme resulting in small modifications of the spatial relationships within the active site. The course of the reaction with other substrates was also examined. The 19-methyl group, very important for the binding, does not influence the proton transfer since the results are identical with **1** and **5**. Some 4β → 10 intramolecular transfer has also been found with a $\Delta^{5(10)}$-3-keto steroid, **4**, showing the flexibility of the active site. Another Δ^5-3-keto steroid, **3**, shows a very different behavior. In this case, the 4β-proton is selectively removed. An enzymic exchange of the 6β-proton is also taking place. Thus the examination of the protons involved in the isomerization and of the intramolecular character of the reaction offers a sensitive method for the study of the interaction in the enzyme-substrate complex.

Isomerases constitute a very important class of enzymes which catalyze either a ketol isomerization (phosphoglucose isomerases,[1] triosephosphate isomerases,[2,3] glyoxalases[4]) or a double bond migration (Δ^5-3-ketosteroid isomerases,[5] prostaglandin isomerases[6]). They represent a rather simple case of acid-base catalysis since they require no cofactor and generally no metal ion and some of them have been extensively studied. In most cases, an at least partially intramolecular stereospecific proton transfer has been demonstrated by using either a labeled substrate in H_2O or a nonlabeled one in D_2O (or ^3H-H_2O).

With the Δ^5-3-ketosteroid isomerase of *P. testosteroni* (EC 5.3.3.1) in which we are interested, this intramolecular transfer first discovered by Talalay et al.[5] was reinvestigated by Malhotra and Ringold,[7] who have shown that the 4β-proton was stereospecifically removed and transferred on carbon 6. They have

(1) Rose, I. A.; O'Connell, E. L. *J. Biol. Chem.* **1961**, *236*, 3086-3092.
(2) Rose, I. A. *Brookhaven Symp. Biol.* **1962**, *No. 15*, 293.
(3) Albery, W. J.; Knowles, J. R. *Biochemistry* **1976**, *15*, 5588-5640.
(4) Hall, S. S.; Doweyko, A. M.; Jordan, F. *J. Am. Chem. Soc.* **1976**, *98*, 7460-7461.
(5) Talalay, P.; Wang, V. S. *Biochim. Biophys. Acta* **1955**, *18*, 300-301.
(6) Jones, R. L.; Cammock, S.; Horton, E. W. *Biochim. Biophys. Acta* **1972**, *280*, 588-601.
(7) Malhotra, S. K.; Ringold, H. J. *J. Am. Chem. Soc.* **1965**, *87*, 3228-3236.

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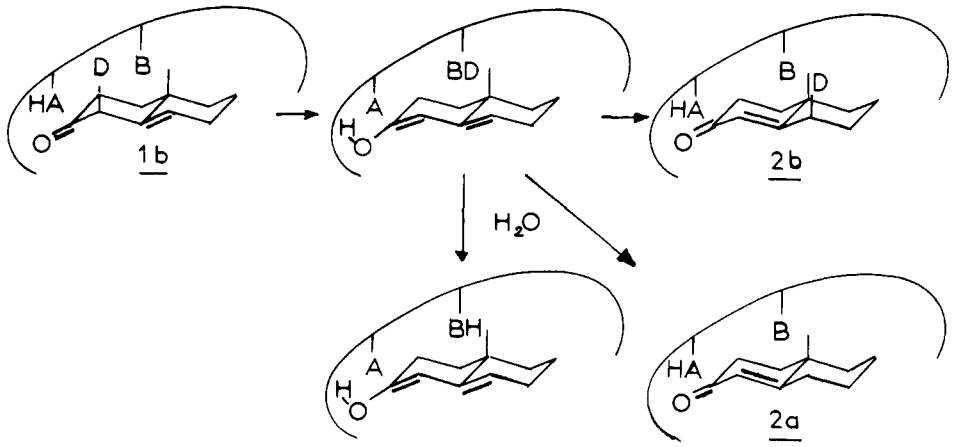


Figure 1. Mechanism of the enzymic isomerization of Δ^5-3-keto steroids.

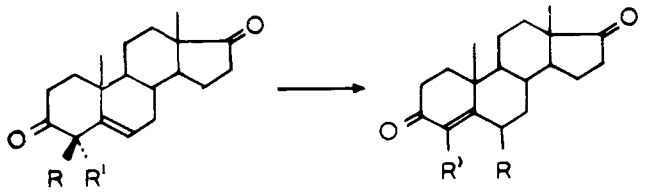
proposed an enolization–reprotonation mechanism depicted in Figure 1 (**1b** → **2b**). This reaction is often quoted in the literature as an example of enzymic specificity.

Indeed, besides the intramolecular transfer, both groups have also observed some exchange with the medium, which can be explained either by an exchange on the basic group (BD → BH or BH → BD) or by direct protonation by the buffer.

We chose to get further information about enzyme–substrate interactions in the active site by considering the extent of 4β → 6β-proton transfer.

This intramolecular migration implies a suitable location of the basic group B with respect to positions 4 and 6: with a "perfect" enzyme–substrate fit only 6β-deuterated product should be found. Otherwise, a less efficient enzyme–substrate interaction could produce a higher percentage of exchange vs. intramolecular migration. We assumed that changes in the enzyme–substrate interactions could thus be revealed either with a given substrate, if conformational changes in the protein are induced by changing the experimental conditions, or by using different substrates.

At the beginning of this work, we first repeated the experiments of Malhotra and Ringold[7] with **1b** as a substrate. As already reported in a preliminary communication,[8] we obtained different results. In the previous work,[7] deuterium was localized by IR, which is not a very accurate method. Using mass spectrometry, we found that **1b**, isomerized under the same conditions, yields **2b** (50%) and **2a** (25%) but also a substantial amount (25%) of **2c** (Table I), implying the elimination of the 4α-proton.



1a R = R' = H
1b R = D R' = H
1c R = H R' = D

2a R = R' = H
2b R = D R' = H
2c R = H R' = D
2d R = R' = D

Thus the two hydrogens on carbon 4 are competitively abstracted. This lack of stereospecificity in an enzymic reaction was rather surprising and led us to further study this interesting problem before completing the work with other substrates.

This isomerization has been studied simultaneously by two other groups, with cholesterol oxidases from bacterial origin.[9] Δ^5-Cholestenone is assumed to be an intermediate in the transformation of cholesterol into Δ^4-cholestenone catalyzed by these enzymes. With the oxidase from *Nocardia erythropolis*, a competitive abstraction of the 4α- and 4β-hydrogens has also been observed[10] whereas with the enzyme of *Brevibacterium sterolicum* a stereospecific removal of the 4β-proton has been reported.[11]

Table I. Isomerization of Androst-5-ene-3,17-dione **1**

substrate	buffer	temp, °C	% D retained or incorporated	% product monodeuterated: carbon 4 (2c)	% product monodeuterated: carbon 6 (2b)
1a	D_2O	25	0.19 (D_1) 0.03 (D_2)[a]	9	10
1a	D_2O	30	0.35 (D_1) 0.16 (D_2)	15	20
1b[b]	H_2O	5	0.93	17	76
1b	H_2O	10	0.84	19	65
1b	H_2O	20	0.80	23	57
1b	H_2O	25	0.75	25	50
1b	H_2O	30	0.67	30	37
1c[c]	H_2O	25	0.93	100	0
1c	H_2O	30	0.84	100	0

[a] The mass spectrum of the product showed that the label is incorporated on carbons 6 (or) 4.[19] The absence of M + 2 peak in the spectrum of the saturated ketone exchanged by NaOH in CH_3OH showed that the product is not dideuterated on carbon 6. The repartition in table I was then derived.[22] [b] **1b** contains more than 0.95% D. [c] **1c** contains 0.89% D.

Results and Discussion

(I) Isomerization of Androst-5-ene-3,17-dione 1. 1. The Removal of the 4α-Proton is an Enzymic Process. Before trying to explain the nonstereospecificity of the reaction, we had to prove that the removal of the 4α-proton was really occurring in the active site. We have already excluded[8] the most trivial explanations, that is the nonstereospecific labeling of the substrate and the spontaneous, nonenzymic isomerization.

Another hypothesis which had to be ruled out was the epimerization **1b** → **1c** in the phosphate buffer before the enzymic reaction, followed by the expected 4β → 6β transfer shown on Figure 1. **1b** was left in phosphate buffer, pH 7, for 5 min and then extracted with benzene. With the ^1H NMR spectrum of the recovered product, we found a slight epimerization (5–10%), due to the buffer since no epimerization occurred in benzene. However, the enzymic isomerization being almost instantaneous in the conditions we used,[12] this epimerization cannot explain the amount of **2c** (25%) found after isomerization of **1b**. This led us to conclude that the enzyme was involved in the abstraction of the

(8) Viger, A.; Marquet, A. *Biochim. Biophys. Acta* **1977**, *485*, 482–487.
(9) The more complex case of isomerases of mammalian origin, which has already raised many discussions, will be considered in a following paper.
(10) Smith, A. G.; Brooks, C. J. W. *Biochem. J.* **1977**, *167*, 121–129.
(11) Nambara, T.; Ikegawa, S.; Hirayama, T.; Hosoda, H. *Chem. Pharm. Bull.* **1978**, *26*, 757–764.
(12) Even if the enzymic reaction required 5 min, the blank observed in the buffer would be overestimated, since the concentration of the substrate is decreasing rapidly in the presence of the enzyme.

4α-hydrogen. But the substrate could also be epimerized by a basic group of the enzyme located out of the active site. This hypothesis was ruled out by the following experiments.

When the deuterated substrate **1b** was incubated with a thermally denatured enzyme in the conditions usually used for the isomerization and then extracted with benzene, the epimerization[13] and deuterium loss (7%) were exactly the same as in the buffer alone.[15]

The enzyme was also inactivated by the k_{cat} inhibitor described by Batzold and Robinson[16,17] to refute the argument that thermal denaturation can change drastically the protein conformation and properties.

The results were the same as those obtained with the blank. Thus we can conclude that we are really dealing with a nonstereospecific proton abstraction during the isomerization process.

2. The Fate of the 4α Hydrogen. In order to examine the fate of the 4α hydrogen, we have synthesized **1c**, deuterated in the 4α-position. As expected, the isomerization occurs with some loss of the 4α-deuterium, which increases with the temperature (Table I). A consistent temperature effect is also observed for the elimination of the 4α proton in the 4β-deuterated substrate (Table I).[18]

The interesting point was to determine if the 4α-deuterium has been transferred onto carbon 6. By mass spectrometry, as previously described,[19] we did not find any label on carbon 6, thus the 4α-proton has been eliminated in the medium (Table I).

3. Isomerization of the Nonlabeled Substrate 1a in D_2O. This study was completed by the conjugation of **1a** in D_2O. Talalay[5] has already demonstrated that there was some deuterium incorporation (0.12 D in 89% D_2O at 25 °C). We observed after lyophilization and resuspension of the enzyme in D_2O buffer a slightly higher incorporation, increasing with the temperature. We found that besides **2b** which is expected through the exchange with the medium some of **2c** and dideuterated molecules **2d** are recovered.[21] This is an important point for the discussion of the mechanism since it is not possible to explain the presence of **2c** or **2d** by the classical scheme reported in Figure 1.

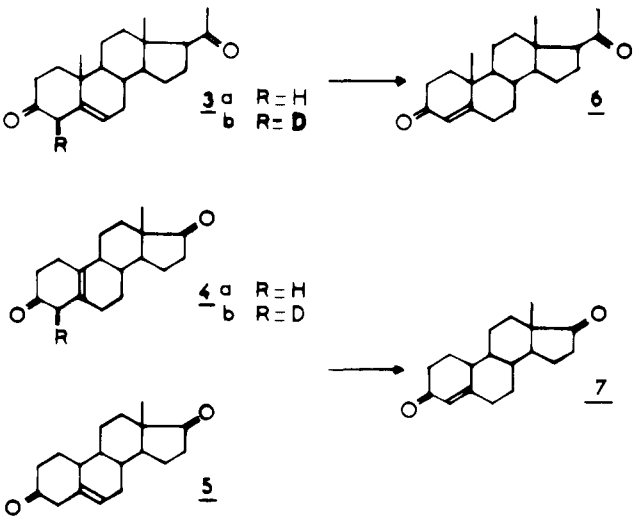


Figure 2. Other isomerase substrates.

Table II. Isomerization of **3**, **4** and **5** at 25 °C.

substrate	buffer	% D retained or incorporated in the product	% product monodeuterated: carbon 4[c]	% product monodeuterated: carbon 6 (or 10)[c]	time
1a	D_2O	0.19 (D_1) 0.03 (D_2)	9	10	5 min
1b	H_2O	0.75	25	50	5 min
3a	D_2O	0.25 (D_1) 0.00 (D_2)	0	25	6 h[d]
3b[a]	H_2O	0.40	0	40	5 h
4a	D_2O	0.32 (D_1) 0.12 (D_2)	20	12	2 h
4b[b]	H_2O	0.27	11	16	30 min
5	D_2O	0.20 (D_1) 0.06 (D_2)	14	6	5 min

[a] **3b** contains more than 0.95% D. [b] **4b** contains more than 0.95% D (4β-D/4α-D = 93/7). [c] The repartition has been calculated as in the preceding cases.[22] See footnote of Table I. [d] **3** is not very soluble in the ethanol–buffer mixture and part of the substrate precipitates at the beginning of the reaction.

4. Influence of the Experimental Conditions on the Reaction Course. Temperature. We have seen that temperature has a strong influence on the course of the reaction, the most important effect being the increased participation of the 4α-proton at higher temperature. The exchange with the medium is also more important, but this phenomenon, already observed by Malhotra and Ringold[7] and in related cases,[1] was expected.

It was also interesting to check the influence of the other factors—pH and percent of cosolvent—which are known to act on the kinetic parameters.[23,24]

Amount of Cosolvent. Weintraub et al.[24] have already shown a decrease in the substrate affinity and a decrease of k_{cat} with increasing concentration of cosolvent. We checked if the composition of the ethanol and buffer mixture also influenced the reaction course: the effect is not spectacular, the amount of deuterium on carbon 4 increases slightly with the amount of ethanol, but the difference remains close to the experimental error[15] (see Experimental Section).

pH. The reaction has been studied at three different pH, using different buffers: pH 5.5 (AcONa–AcOH, 0.03 M); pH 7 (HPO_4^{2-}, $H_2PO_4^-$, 0.03 M); pH 8.5 (Tris–HCl, 0.03 M). Within these values the spontaneous isomerization remains negligible. The

(13) In this experiment the deuterium stereochemistry could not be determined directly by ^1H NMR since the crude extraction product is not pure enough. It was checked, after reduction, by thiobenzoylation of the diol followed by photolysis as already described.[14]

(14) Viger, A.; Coustal, S.; Marquet, A. *Tetrahedron*, **1978**, *34*, 3285–3290.

(15) The experimental error on a mass spectrometric determination with a UV recorder is assumed to be 3%.

(16) (a) Batzold, F. H.; Robinson, C. H. *J. Am. Chem. Soc.* **1975**, *97*, 2576–2578. (b) Batzold, F. H.; Robinson, C. H. *J. Org. Chem.* **1976**, *41*, 313–317. (c) Carrell, H. L.; Glusker, J. P.; Covey, D. F.; Batzold, F. H.; Robinson, C. H. *J. Am. Chem. Soc.* **1978**, *100*, 4282–4289.

(17) The denaturation was accomplished by a 200 mM solution of the acetylenic substrate in dioxane.[16a] We never suppressed completely the activity even by adding more k_{cat} substrate and by leaving the enzyme for 1 week in contact with this inhibitor. The origin of this remaining activity is not clear and may be due to a nondetected impurity. But this activity is not disturbing since the isomerization is slowed down by isotopic effects and in the same conditions of concentration **1b** is not isomerized by this residual activity.

(18) There is of course no complementarity at a given temperature between the amount of deuterium remaining on carbon 4 with **1b** and lost with **1c** because of the isotopic effects. Since these isotopic effects are not known accurately, we did not attempt any quantitative correlation.

(19) The total amount of deuterium is determined by mass spectrometry. The fragmentations of **2** show that the label is located on carbons 4 and (or) 6, but it is impossible to determine the exact repartition.[20] The amount of deuterium on carbon 6 (x) is determined by mass spectrometry after reduction of the double bond and selective exchange of the 4-position;[8] the amount of deuterium on carbon 4 (y) is then easily calculated (total deuterium $- x$). Since the accuracy on x is 3%, the accuracy on y is also 3% $\Delta(x + y)/(x + y) = 3/100$ (1); $\Delta x/x = 3/100$ (2). Combination of eq 1 and 2 gives $\Delta y/y = 3/100$.

(20) Viger, A.; Marquet, A.; Tabet, J. C. *Org. Mass. Spectrom.* **1976**, *11*, 1063–1076.

(21) The reported incorporations are due to the enzymic isomerization: we checked that there is no further exchange of the product **2** (no incorporation of deuterium in **2a** in D_2O under the same conditions and no deuterium loss with **2b** in H_2O).

(22) If the conjugated product contains x monodeuterated and y dideuterated molecules, the total deuterium incorporated is $x + 2y$. If z is the amount of deuterium remainining after reduction and exchange, the deuterium incorporated on carbon 4 is given by $x + 2y - z$.

(23) (a) Kawahara, F. S.; Talalay, P. *J. Biol. Chem.* **1960**, *235*, 1. (b) *Ibid.* **1962**, *237*, 1500–1506. (c) Wang, S. F.; Kawahara, F. S.; Talalay, P. *Ibid.* **1963**, *238*, 576–585.

(24) (a) Weintraub, H.; Alfsen, A.; Baulieu, E. E. *Eur. J. Biochem.* **1970**, *12*, 217–221. (b) Weintraub, H.; Baulieu, E. E.; Alfsen, A. *Biochim. Biophys. Acta* **1972**, *258*, 655–672.

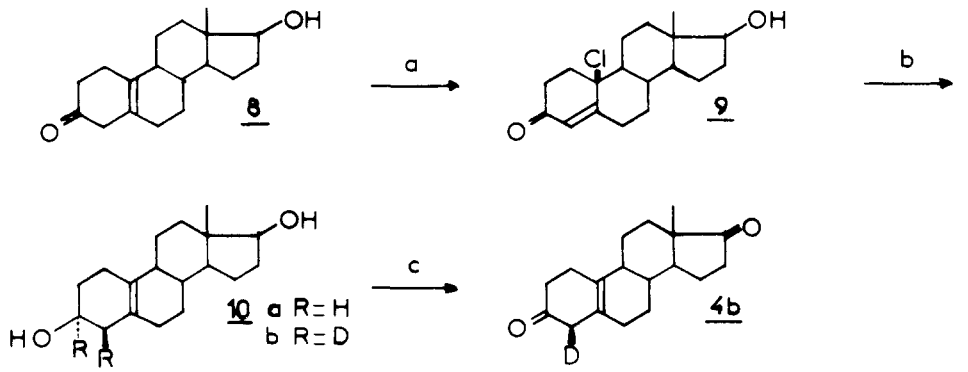


Figure 3. Synthesis of **4b**: (a) NCS, (b) $LiAlD_4$, (c) Jones reagent.

results are identical for the three experiments and the reaction course is then pH independent. This shows that the reaction does not depend on the nature of the buffer nucleophiles and strengthens the conclusion that the nonstereospecific abstraction of 4α- and 4β-protons is an enzymic process.

(II) Isomerization of Other Substrates. Since the enzyme can isomerize various $\Delta^{5(6)}$- or even $\Delta^{5(10)}$-3-keto steroids, it was interesting to investigate how the proton is transferred with these substrates.

Indeed, we had to limit our study to compounds **3–5** (Figure 2). The other Δ^5-3-keto steroids available to us have no 17-polar group and are not substrates of the enzyme in the conditions usualy used.[25]

The isomerization of **3a**, **4a**, and **5** has been studied according to the methodology previously developed for **1a** in D_2O buffer.

The 4β-deuterated derivatives **3b** and **4b** were also synthesized. The preparation of **3b** was achieved by the method used for **1b**, starting from 3β,17β-dihydroxypregn-5-ene dibenzoate (see Experimental Section).

4b was obtained by a modified procedure (Figure 3), the preceding scheme leading to a complex mixture.[53] **9** was prepared according to Ringold[27] and transformed with $LiAlD_4$ into **10b** (identified with an authentic sample of **10a**).[28] We suppose that the deuterium is located in the 4β-position by assuming an intramolecular S_N2' reaction initiated by the reduction of the 3-keto group. (We have already demonstrated[14] that in the case of 6β-chloro-17β-hydroxyandrost-4-ene-3-one this reaction occurs with syn stereochemistry.) As already pointed out,[14] the oxidation of the βγ-ethylenic alcohols is not easy to carry out and the isolation of pure βγ-ethylenic ketones rests on the possibility of selective precipitation. In this case, the oxidation step is slowed down by the isotopic effects due to the deuterium atom introduced in 3α. Because of the longer reaction time, more conjugated and secondary products are produced, preventing precipitation of pure **4b**. **4b** was recovered by extraction and silica gel chromatography. The deuterium stereochemistry in **4b** was checked by ^1H NMR at 270 MHz.[52] Some epimerization has occurred in the purification step, as we have already observed in the androstane case.[14] But it was low (7%), and this sample was used for the enzymic reactions.

The results of the enzymic isomerization of **3–5** are reported in Table II. The data concerning **1a** and **1b** are included for comparison. Deuterium has been localized by mass spectrometry after reduction of the double bond and alkaline exchange as previously described for **1**.[19]

Even if the incorporation patterns for **1a** and **5** are very similar, the reaction course is highly dependent on the substrate nature. Compound **3** has a very different behavior: in the case of **3b** the exchange with the medium is more important than with **1b** where 60% of the label is lost during the isomerization. Another important feature of this reaction is that all the remaining deuterium has been transferred intramolecularly on carbon 6, and no deuterium is left on carbon 4. The isomerization of **3a** in D_2O yields a product deuterated on carbon 6, and no deuterium is incorporated on carbon 4.

In the case of **4b**, the exchange with the medium is also very important (70% of the initial deuterium is lost). Some deuterium (16%) is also transferred by an intramolecular mechanism, and 11% remains on carbon 4. With **4a** a high incorporation value and some dideuterated species are found, in agreement with the important exchange detected in the case of **4b**.

It was first necessary to check that these results were due to the enzymic isomerization and not to reactions occurring in the buffer before or after conjugation. This was done as previously described for **1b**: **3b** and **4b** were left in the buffer during the time required for the enzymic reaction respectively 30 min and 5 h and then recovered and analyzed by ^1H NMR. **4b** shows no loss of deuterium but 9% epimerization, while with **3b** only 3–5% of exchange and (or) epimerization are observed.

On the other hand, the nonlabeled conjugated products **6** and **7** were incubated with the enzyme in D_2O buffer. There is no incorporation of deuterium in the case of **7**, but with **6** 0.4 deuterium is incorporated on carbon 6. This is an enzymic process since no deuterium is introduced without enzyme. Since we did not find any dideuterated species, we can conclude that only one of the two protons on carbon 6 is exchanged. (We did not determine which one, but it is likely the 6β-proton.)

(III) Discussion. 1. Isomerization of Androst-5-ene-3,17-dione 1. Figure 4 shows the different pathways which may be involved in the isomerization of **1b**.

The formation of **2b** and **2a** is in agreement with the previously reported results. The presence of **2b** confirms the 4β → 6β transfer[29] (path a). **2a** can be produced either through direct protonation of the enol by the solvent (path b) or after exchange BD → BH on the enzyme (path a). But we have also detected a competitive pathway leading to a conjugated ketone labeled on carbon 4 (**2c**), indicating a competitive abstraction of the 4α- and 4β-protons. We ruled out a 4β → 6β transfer on a substrate epimerized out of the active site (either by the buffer or through

(25) To explain this result, Jones[26] has postulated a micellar aggregation of these compounds. They become good substrates when the micelles are disaggregated by increasing the amount of cosolvent. We did not perform experiments under such conditions since they are so different from those used with the other compounds that any accurate comparison becomes impossible.

(26) (a) Jones, J. B.; Wigfield, D. C. *Can. J. Chem.* **1968**, *46*, 1459–1465. (b) Jones, J. B.; Gordon, K. D. *Biochemistry* **1973**, *12*, 71–76.

(27) Mukawa, F.; Dorfman, R. I.; Ringold, H. J. *Steroids* **1963**, *1*, 9–20.

(28) Levine, S. G.; Eudy, N. H.; Leffler, C. F. *J. Org. Chem.* **1966**, *31*, 3995–4002. We have prepared **10a** as described, but we found a different melting point (150 °C instead of 130 °C). However, the ^1H NMR spectrum is identical with the published one.

(29) The method used in this study does not allow us to determine the stereochemistry on carbon 6. We have checked by ^1H NMR that the deuterium is principally transferred in 6β: the 4-C vinyl proton which appears as a single peak is markedly sharpened in the case of 6β-deuterated analogue by the disappearance of the allylic coupling,[30] Malhotra and Ringold[7] have located the deuterium in the conjugated product by IR,[31] which is not a very accurate method (the band intensity is very weak). We think that both techniques are not precise enough to rule out the presence of some 6α-deuterium.

(30) Wittstruck, T. A.; Malhotra, S. K.; Ringold, H. J. *J. Am. Chem. Soc.* **1963**, *85*, 1699–1700.

(31) Malhotra, S. K.; Ringold, H. J. *J. Am. Chem. Soc.* **1964**, *86*, 1997–2003.

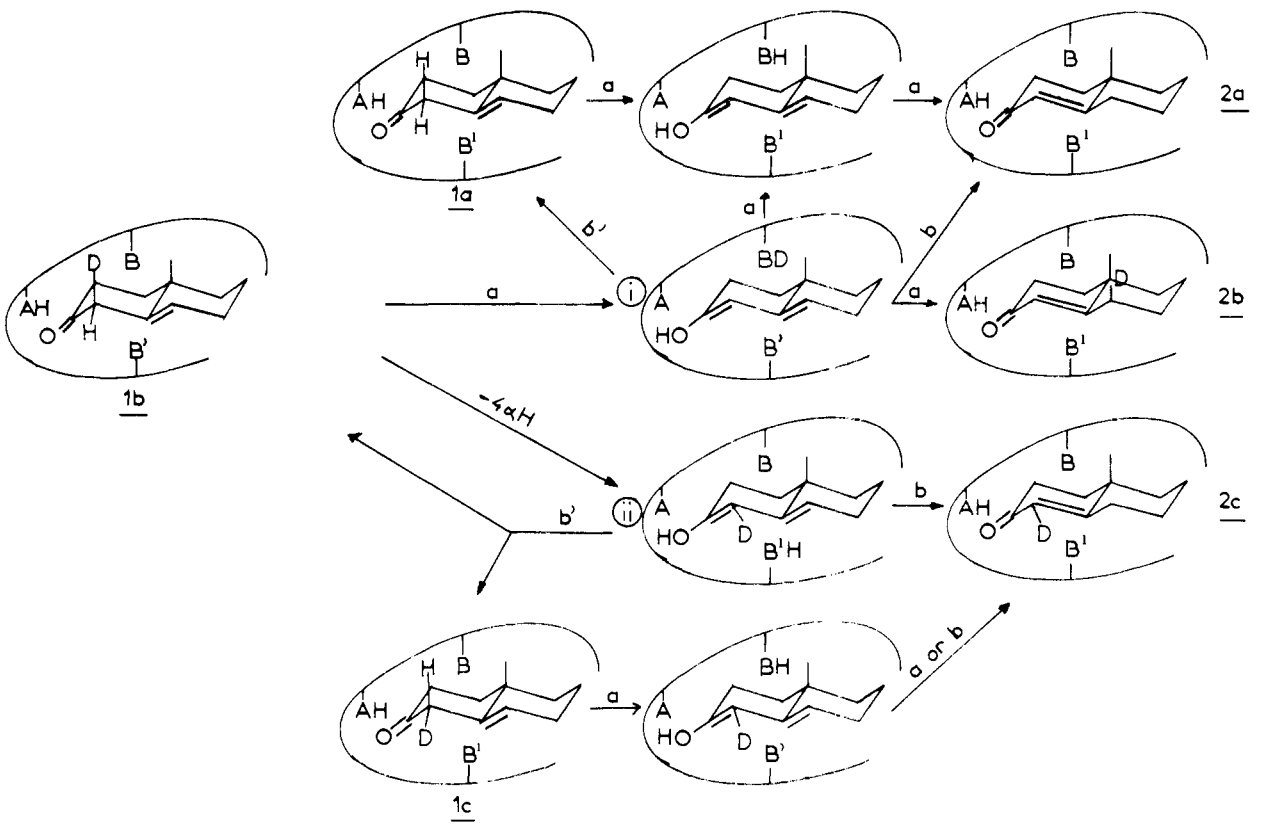


Figure 4. Isomerization of **1b** in H_2O: the abstraction of the 4α- and 4β-protons may be a reversible step, but we have no possibility to check this point.

a basic group of the protein). We conclude that the 4α-proton is removed in or inside of the active site, during the catalytic process, by either another base B' or a solvent molecule. We have also shown that the 4α proton is not transferred onto carbon 6.

The 4$\beta \rightarrow$ 6β transfer, accompanied by some exchange during the reprotonation of the enol (paths a + b), may remain the exclusive route for the conjugation if it occurs on a substrate partially epimerized in the active site, through reprotonation on carbon 4 (path b') after elimination of the 4α proton.[32]

But the formation of **2c** can as well be rationalized by the direct protonation of the deuterated enol ii by the solvent (path b).

The experiments in D_2O should help to clarify this point. The isomerization of **1a** in D_2O yields, as expected according to the simple picture of Figure 1, **2a** and **2b**.[33] But some species monodeuterated on carbon 4 (**2c**) or dideuterated on carbons 4 and 6 (**2d**) are also found. The amount of deuterium on carbon 4 at 25 °C and at 30 °C is respectively 0.12 D and 0.31 D.

This proves that during the isomerization, the reprotonation of the enol on carbon 4 takes place, thus strengthening the above hypothesis of a reaction occurring on an epimerized substrate. A nonstereospecific reprotonation would produce **1c** and **1b**, leading respectively to **2c** + **2d** or **2d** (Figure 5).

We did not try to find a quantitative correlation between the results obtained in D_2O and H_2O since it is well-known that changing the solvent from H_2O to D_2O may change the conformation of the enzyme[35] and the enzyme–substrate interactions by modifying the hydrogen bond lengths, the hydrophobic interactions, ...[36] We have also no criteria for asserting that all mobile enzyme protons have been completely exchanged by lyophilization and resuspension in D_2O. On the other hand, a quantitative discussion should take into account three different isotopic effects, the primary isotopic effects for the 4α- and 4β-deuterated substrates and the solvent isotopic effect. The first ones are not easy to determine accurately because of the competitive abstraction of the 4α- and 4β-protons. A determination of the solvent isotopic effect requires highly precise kinetic measures[37] that we did not undertake.

Although some doubtful points remain, some important features come out. It is now clearly established that this enzymic mechanism is not univocal and involves an intramolecular migration yielding **2b**, a 4α participation yielding **2c**, and some exchange with the medium, yielding **2a**. It is therefore possible to evaluate the relative contributions of each process by measuring the relative percent of **2a**, **2b**, and **2c**. This provides *a very sensitive probe to study the enzyme substrate interactions*: if the basic group B (Figures 4 and 5) is suitably located between carbons 4 and 6, the intramolecular migration should be favored and a substantial amount of **2b** should be found. If some unfavorable conformational changes occur in the protein resulting in a less efficient enzyme–substrate interaction, the percentage of **2b** vs. **2a** and **2c** is expected to decrease.

That is why we have tested if the experimental conditions which influence the kinetics of the reaction also influence the proton transfer. If no significant variation was observed by changing the pH or the amount of cosolvent, the reaction course was found to be highly temperature dependent (Table I). The increase of the 4α participation with the temperature can indeed be attributed

(32) The recovery of epimerized substrate if the reaction is stopped before completion would support this hypothesis. It should be possible to detect the epimerized substrate before its isomerization since the dissociation rate of the enzyme substrate complex is of the same order of magnitude as the rate of product formation.[7] This experiment has been done, and the deuterium distribution in the remaining substrate was analyzed in the usual way. The amounts of **1a** and **1c** are very close to those observed in the buffer alone (see Experimental Section) and do not allow a clear conclusion.

(33) Recently, Benisek et al.[34] have reported that the isomerization of androst-5-ene-3,17-dione by the isomerase from *Pseudomonas putida* yielded 48% of nondeuterated, 48% of monodeuterated, and 4% of dideuterated product. These results are very close to those obtained with the isomerase from *Pseudomonas testosteroni*.

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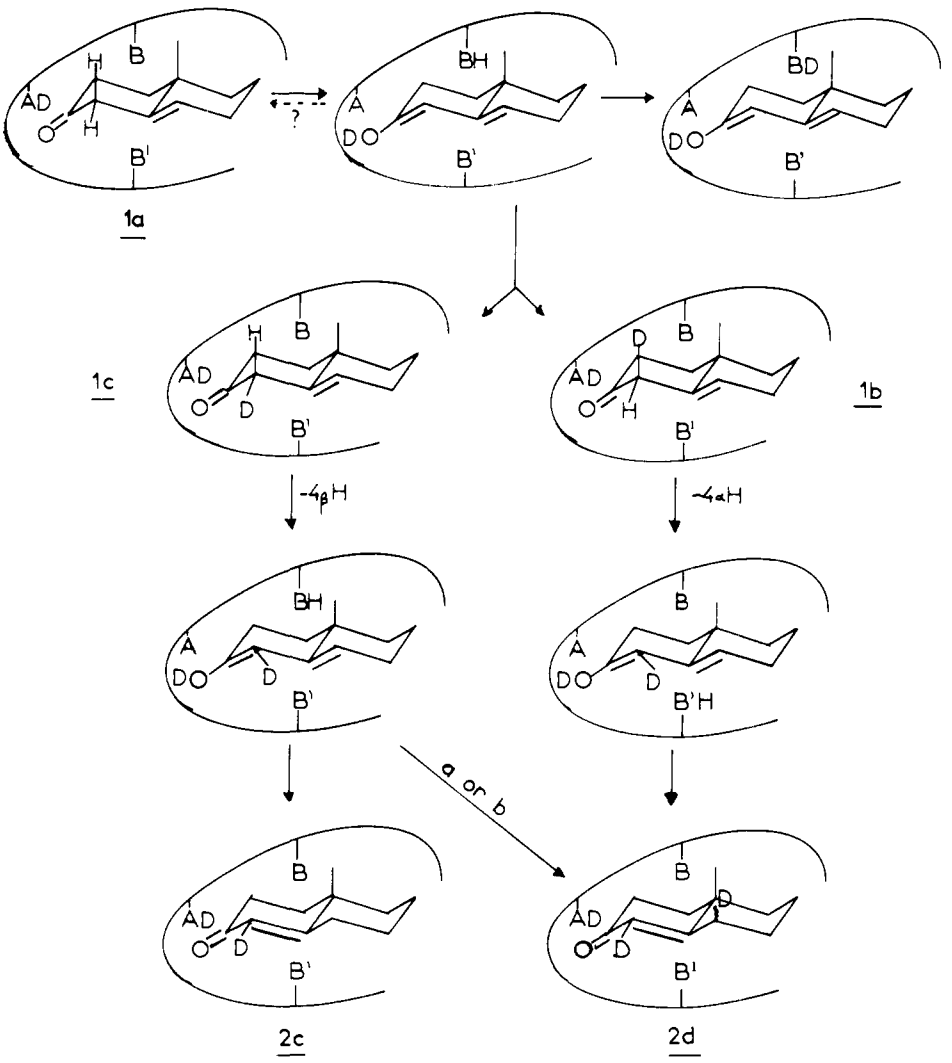

Figure 5. Isomerization of **1a** in D_2O: formation of **2c** and **2d**.

to some conformational changes in the protein.

2. Isomerization of Other Substrates. We have also examined the course of the reaction with other substrates in order to detect changes in the enzyme–substrate relationships.

Estr-5-ene-3,17-dione 5. This compound was studied to test the influence of the 19-methyl group. It has been reported that this methyl group has a great influence in the substrate binding at the active site: K_M = 83 μM for **5** and 310 μM for **1a**.[38] The 19-nor inhibitors bind also more tightly than their analogues with a 19-methyl group[38] (for example K_I = 200 μM for **2a** and 60 μM for 7). Carrell et al.[16c] have suggested that the 19-methyl group may play a role in the enzymic reaction by guiding the proton to the appropriate site for protonation. However the k_{cat} of **5** and **1a** are of the same order of magnitude (14×10^4 s^{-1} in 3.3% of methanol for **1a** and 5.4×10^4 s^{-1} for **5**),[39] and we see here that **5** and **1** incorporate about the same amount of deuterium in D_2O. This shows that the 19-methyl group has no drastic influence on the course of the proton transfer.

Estr-5(10)-ene-3,17-dione 4. **4** was a priori interesting to study. The double bond at the AB ring junction is in a very different location, and it is a much slower reacting substrate. It was worthwhile to find out if the intramolecular migration is still taking place in this case.

An important exchange with the medium is observed (70% of the initial deuterium is lost with **4b** and 0.56 deuterium is incorporated in D_2O). It occurs during the isomerization step since no further exchange is observed in the conjugated product.

Some product deuterated on carbon 4 is also recovered after isomerization of **4b** in H_2O, but the small amount of deuterium remaining in the product does not allow a precise discussion. Referring to our extensive study of **1** and to the qualitative agreement between the results with **1** and **4**, we think that the 4α- and 4β-protons are also competitively removed in **4**. The important incorporation of deuterium on carbon 4 when the reaction is performed in D_2O is consistent with this conclusion.

However there is some intramolecular transfer, and that is the most important point. It means that the enzyme basic group which is involved in the intramolecular transfer between carbons 4 and 6 in **1** is also able to promote a similar transfer between carbons 4 and 10. The active site is likely flexible since the enzyme can accommodate highly different substrates and isomerize them in an at least partially intramolecular way. The intramolecular migration, however, accounts for only 16% of the reaction instead of 50% in the case of androst-5-ene-3,17-dione **1**, indicating that the basic group of the enzyme which removes the 4β-proton is less suitably located between carbons 4 and 10. The competing processes are then favored.

Pregn-5-ene-3,20-dione 3. The situation is rather different with **3** where the reaction pattern presents important differences, that is the stereospecific abstraction of the 4β-proton and the enzymic exchange of the 6β-proton. This can be explained either by the intrinsic properties of the two substrates related to differences of conformation or by changes in the enzyme substrate relationships.

Although keeping in mind the difficulty to compare crystal and solution conformations, we looked at the X-ray structures of **1** and **3**. The structure of **1** was already known;[16c] we have resolved

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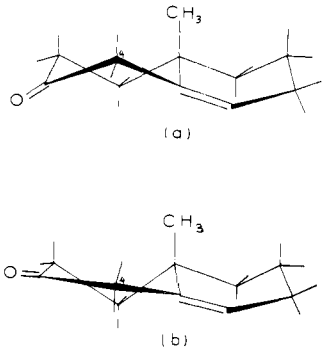

Figure 6. Side views of (a) pregn-5-ene-3,20-dione and (b) androst-5-ene-3,17-dione.

that of **3**.[40] In **1** carbons 3–6 are coplanar and the 4α- and 4β-protons are symmetrically located with respect to the plane of the 3-keto group. In **3**, the 4α- and 4β-protons are no longer equivalent and occupy equatorial and axial positions (Figure 6).

It was attractive to assume that the enzymic reaction is sensitive to stereoelectronic control,[41] implying that in **3** the axial proton is stereospecifically removed whereas in **1** the equivalent 4α- and 4β-protons are competitively abstracted.

However the NMR spectra of **1** and **3** do not confirm the differences found in the solid. The J(gem) coupling constants of the 4α- and 4β-protons should be sensitive to the conformation of ring A. According to the Barfield–Grant rule,[42] the π contribution to the geminal coupling constant ($J(\pi)$) varies with the angle between the methylene group and the adjacent π bond (ϕ). If they had the same conformation as in the crystal, $J(\pi)$ for **1** and **3** should be respectively about 4.5 and 1.5 Hz corresponding to the dihedral angles (ϕ) of 30 and 60°. But the observed values are for both compounds 17 ± 0.5 Hz, they have thus probably the same conformation in solution.

Hence the stereoselectivity of proton removal is very likely related to the position of the substrate with respect to the catalytically active groups.

Another important observation is that the 6β-proton is easily exchanged when the conjugated product **6** is treated with the enzyme. Since some exchange occurs on carbon 6 after the isomerization step, it means that in this case the percentage of intramolecular migration deduced from the amount of deuterium recovered in the product is largely underestimated.

This exchange was not observed with the other substrates. Talalay et al.[23c] have already reported that **2** does not incorporate any tritium when incubated in ^3H–H_2O for 1 h with about the same amount of enzyme as we used, but the incorporation reaches 0.82 T when the amount of enzyme is increased by 10^4. It is thus clear that the 6β-proton is abstracted by the enzyme much more rapidly in progesterone **6** than in androst-4-ene-3,17-dione **2**.[43] This can also be explained by slight differences in the positions of the two substrates within the active site.

Conclusion

Our study of the fate of the 4- and 6-hydrogens which are involved in the isomerization has shed some further light on the mechanism of Δ^5-3-ketosteroid isomerase from *Pseudomonas testosteroni*.

We have shown that several processes could compete, the relative contributions of which being very likely related to differences in the enzyme–substrate relationships.

This contribution to the understanding of the mode of action of this enzyme is to be added to many other interesting papers discussing the topology of the active site,[38] the nature of the catalytically active groups,[23,24,44] or the association state.[45–47]

Experimental Section

Melting points were determined on a Köfler melting point apparatus and are uncorrected. NMR spectra were recorded on a Varian T60 or HA-100 spectrometer using Me_4Si as the internal reference. The results are given in δ (ppm), multiplicity, protons number, and J.

Mass spectra were obtained with an AEI MS30 mass spectrometer.

General Procedure for the Enzymic Isomerization. The enzyme was purified by affinity chromatography according to a previously described method[48] with a minor modification: the 19-nortestosterone acetate was bound to agarose by glutathione.[49]

A 10-mg sample of **1b** (0.95 D, determined by ^1H NMR) dissolved in 10 mL of ethanol was added to 350 mL of phosphate buffer, pH 7 (0.03 M), at 25–26 °C, followed by 1000 units of pure bacterial enzyme. The reaction was monitored at 248 nm. When the A_{248} was constant and has reached its theoretical value (1 min is generally enough), the product was extracted with EtOAc, washed, dried, and purified by thin-layer chromatography (EtOAc–hexane, 1:1). The total amount of deuterium in **2** was determined by mass spectrometry.

2 was then reduced by H_2 with 10% Pd/C. After 30 min at room temperature, the product was filtered, washed, and dried. Mass spectrometry analysis proved that there was no deuterium loss during the reduction. The deuterium on carbon 4 was then selectively exchanged by treatment of the reduced compound with NaOH in refluxing methanol for 90 min. After extraction with EtOAc and usual workup the product was purified by chromatography on silica gel (EtOAc–hexane, 1:1). The amount of deuterium remaining on carbon 6 was then determined by mass spectrometry.

Conditions Used for the Isomerization of the Other Substrates. 6b: 10 mg in 50 mL of ethanol in 700 mL of phosphate buffer, followed by 1000 units of enzyme; reaction time was 5 h.

7b: 15 mg in 15 mL of ethanol in 520 mL of phosphate buffer, followed by 12 000 units of enzyme; reaction time was 30 min.

1a: 10 mg in 10 mL of EtOD in 50 mL of phosphate buffer–D_2O, followed by 1000 units of enzyme lyophilized and resuspended in D_2O; reaction time was 1 min.

6a: 10 mg in 20 mL of EtOD in 50 mL of phosphate buffer–D_2O, followed by 3000 units of enzyme in D_2O; reaction time was 6 h.

7a: 14 mg in 10 mL of EtOD in 50 mL of phosphate buffer–D_2O, followed by 3500 units of enzyme in D_2O; reaction time was 2 h.

Isomerization of 1b with Increasing Amounts of Cosolvent. When being isomerized with 3% ethanol, **1b** yielded 25% **2a**, 50% **2b**, and 25% **2c**, and when being isomerized with 17% ethanol, it yielded 35% **2a**, 43% **2b**, and 22% **2c**.

Incubation of 1b in Phosphate Buffer pH 7 without Enzyme. A 20-mg sample of **1b** (0.95 D) dissolved in 20 mL of EtOH was added to 700 mL of phosphate buffer (0.03 M) and left 5 min at 25 °C (A_{248} = 0).

The product was then extracted with benzene, and usual workup yielded 22.4 mg of crude product which was dissolved in 2 mL of THF and added to 198 mg of $LiAlH(O(t\text{-}Bu))_3$ in 2 mL of THF. After 30 min at room temperature 1.5 mL of acetaldehyde was added followed by 10 mL of 5% acetic acid. The mixture was then extracted with EtOAc, giving 36 mg of crude product which afforded after chromatography on silica gel (benzene–EtOAc, 8:2) 19 mg of 3β,17β-dihydroxyandrost-5-ene **11** (0.95 D). The deuterium stereochemistry in **11** has been determined according to the method developed by Barton[50,51] that we have already used in the resolution of a similar problem.[14] The amount of epimerized deuterium is 6%.

Incubation of 1b with the Thermally Inactivated Enzyme. A 20-mg sample of **1b** (0.95 D) in 20 mL of EtOH was incubated with 1000 units of thermally inactivated enzyme (70 °C for 3 days) for 5 min, reduced, and treated as previously described.[14] The amount of epimerized deuterium is 7%.

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Incubation of 1b with the Enzyme Inactivated by the "k_{cat}" Inhibitor. A 1000-unit sample of pure bacterial enzyme was inactivated[16a] by a solution of **3** in dioxanne.[17] After incubation with this inactivated enzyme for 5 min, **1b** was reduced and treated as previously described.[14] The amount of epimerized deuterium is 7%.

Isomerization of 1b with a Limiting Amount of Enzyme. A 30-mg sample of **1b** (0.95 D) was dissolved in 30 mL of EtOH and then added to 1050 mL of 0.03 M phosphate buffer, pH 7. A 12-unit sample of pure enzyme was then added in order to isomerize 40% of the mixture of **1** and **2**. It is not possible to purify this mixture by chromatography since the label may be epimerized during this step.[14] The Δ^5-3-keto group in **1** was selectively reduced with a limited amount of $LiAlH(O(t\text{-}Bu))_3$, yielding 3β-hydroxyandrost-5-ene-17-one which could be easily separated from **2**. After reduction of the 17-keto group, the 3β,17β-dihydroxyandrost-5-ene was treated as previously described—12% of the initial deuterium has been exchanged and 18% of the remaining deuterium has been epimerized.

Incubation of the Other Substrates in Phosphate Buffer without Enzyme. 3b (25 mg) dissolved in 100 mL of EtOH and 1500 mL of 0.03 M phosphate buffer was left for 5 h at 25 °C. After extraction with benzene, the ^1H NMR spectrum showed a slight exchange (6%).

4b (8 mg) in 8 mL of EtOH and 280 mL of 0.03 M phosphate buffer, *p*H 7, was left for 30 min at 25 °C. After extraction with benzene and chromatography on silica gel the ^1H NMR spectrum showed a slight epimerization (9%).

10β-Chloro-17β-hydroxyestr-5-ene-3-one (9). **9** was prepared according to ref 27 (47%): mp 144 °C; NMR ($CDCl_3$) δ 5.7 (m, 1 H, 4-H), 3.55 (t, 1 H, 17 α-H), 0.82 (s, 3 H, 18-Me).

3α,4β-Dideuterio-3β,17β-dihydroxyestr-5(10)-ene (10b). A 470 mg sample of **9** dissolved in 10 mL of a 1:1 mixture of ether–benzene was added to 470 mg of $LiAlD_4$ in 2 mL of anhydrous ether and heated to reflux for 2 h. After one further hour at room temperature the mixture was hydrolyzed with EtOAc, diluted with HCl and extracted with EtOAc, yielding 380 mg of crude product, which was purified by chromatography on silica gel (benzene–EtOAc, 8:2). A 270-mg sample of pure **10b** was obtained (64%): mp 150 °C. Mass Spectrometry: D_2, 0.95, D_1, 0.05. NMR ($CDCl_3$): δ 3.55 (t, 1 H, 17α-H), 0.88 (s, 3 H, 18-Me), no olefinic proton.

4β-Deuterioestr-5(10)-ene-3,17-dione (4b). A slow stream of N_2 was bubbled in 0.79 mL of Jones reagent for a few minutes, and this reagent was added to 130 mg of **10b** in 30 mL of acetone previously distilled on $KMnO_4$. After 15 min at 0 °C, 0.66 mL of 2-propanol was added. The mixture was then poured into iced water and extracted with benzene, yielding 84.7 mg of crude product. Purification on silica gel (benzene–EtOAc, 95:5) yielded 12 mg of **4b** (10%).

NMR (270 MHz):[52] amount of incorporated deuterium $\geq$ 0.95 (no A.B. signal corresponding to the 4α- and 4β-protons), large singlet at 2.70 ppm, little shoulder at 2.64 ppm (relative areas 93/7); 93% of the deuterium has the 4β stereochemistry.

5,6-Oxido-3β,20β-dihydroxypregnane Dibenzoate (12). A solution of 7.1 g of 3β,20β-dihydroxypregn-5-ene dibenzoate (mp 226 °C) and 5.8 g of *m*-chloroperbenzoic acid in 80 mL of methylene chloride was left overnight at room temperature. The mixture was washed with aqueous $NaHCO_3$ and water, dried, and concentrated in vacuo to yield 6.3 g of a mixture of the 5α,6α- and 5β,6β-epoxides. This mixture could not be purified by conventional chromatographic treatments.

6β-Chloro-3β,5α,20β-trihydroxypregnane 3,20-Dibenzoate (13a). A 10.3-mL sample of concentrated HCl (37%) was added to a solution of 6.3 g of **12** in 250 mL of acetone. The solution was allowed to stand for 30 min at room temperature and slowly diluted with water to yield 6.6 g of a mixture of chlorhydrins which were purified by chromatography on silica gel (hexane–EtOAc, 2:1) to yield 4.4 g of **13a** (65%), mp 140 °C.

NMR ($CDCl_3$): δ 0.75 (s, 3 H, 18-Me), 1.3 (s + d, 6 H, 21-Me + 19-Me), 3.9 (m, 1 H, 6α-H), 5.3 (m, 2 H, 3α- + 20α-H). This isomer was identified by comparison of the chemical shifts of the 3α- and 6α-protons with those obtained in the chlorhydrin described by Malhotra and Ringold[7] in the synthesis of **1b**.

6β-Chloro-3β,20β-dihydroxypregn-4-ene Dibenzoate (14). A 8-mL sample of $SOCl_2$ was added dropwise to an ice-cooled solution of 4 g of chlorhydrin **13a** in 80 mL of dry pyridine. After being left to stand for 30 min at 0 °C, the solution was poured into crushed ice and the product was filtered off, washed, and dried. Recrystallization (acetone) afforded pure material, mp 216 °C (80%).

NMR ($CDCl_3$): δ 0.75 (s, 3 H, 18-Me), 1.25 (d, 3 H, 21-Me, J = 7 Hz), 1.35 (s, 3 H, 19-Me).

4β-Deuterio-3β,20β-dihydroxypregn-5-ene (15). A mixture of 1 g of **14** and 380 mg of $LiAlD_4$ in 20 mL of anhydrous benzene and ether was boiled under reflux with N_2 for 2 h and then stirred overnight at room temperature. The excess of hydride was decomposed by addition of a saturated solution of Na_2SO_4 and the product isolated by extraction with EtOAc. One crystallization (acetone) yielded 402 mg of **15** (71%), mp 202 °C (identical with the nondeuterated compound). M^+ = 319 (1 D).

4β-Deuteriopregn-5-ene-3,20-dione (3b). A 0.8-mL sample of 8 N Jones reagent was added with stirring to an ice-cooled solution of 120 mg of **15** in 40 mL of acetone. After the solution was stirred for another 4 min, 0.4 mL of 2-propanol was added, the resulting solution was filtered on a cotton plug, ice water was added, and nitrogen was allowed to bubble into the solution. The product was then filtered, washed, and dried in vacuo, yielding 60 mg of **3b**.

NMR ($CDCl_3$): δ 0.6 (s, 3 H, 18-Me), 1.13 (s, 3 H, 19-Me), 2.08 (s, 3 H, 21-Me), 2.76 (s, 1 H, 4α-H), 5.33 (m, 1 H, 5-H).

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(52) The 4α- and 4β-protons are not differentiated at 100 MHz, but they appear as an AB system at 2.64 and 2.71 ppm (J = 20 Hz) at 270 MHz.

(53) **Note Added in Proof:** From this complex mixture, a small amount of estr-5-ene-3,17-dione containing 1 D per molecule could be isolated. However, the NMR spectrum showed that the deuterium was distributed on several carbon atoms. The kinetic experiments reported by H. Weintraub et al. (*Biochem. J.* **1980**, *185*, 723–732), without our agreement, were carried out on this preliminary sample and should be run again.

Communications to the Editor

Transition-Metal Binding Site of Bleomycin. Cobalt(III) Bleomycin

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The study of the metal binding properties of the glycol peptide antibiotic bleomycin (**1**) is an active area of research.[1] Although certain radioactive metal complexes of the drug have been synthesized and used as diagnostics in nuclear medicine for some time,[2] the recent realization that an iron ion cofactor is very likely required for the in vivo anticancer activity of bleomycin[3] has stimulated renewed and intense interest in the metal binding properties of the drug. In vitro as well as in vivo experiments with

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