Enzymic isomerization of Δ^5-3-ketosteroids

Sterols containing the Δ^5-3β-ol grouping are biosynthetic precursors of hormonally active Δ^4-3-ketosteroids[1,2]. Chemical[3] or enzymic [1,2,4,5] oxidation of Δ^5-3β-hydroxysteroids leads to the isolation of the corresponding Δ^4-3-ketosteroids. The enzymic conversion involves a primary oxidation of the 3β hydroxyl group by a stereospecific DPN^+-linked β-hydroxysteroid dehydrogenase[5] followed by a rearrangement of the double bond. We wish to present evidence for the existence of a widely distributed steroid isomerase, an enzyme which is distinct from the oxidizing enzyme, and which catalyzes the migration of the double bond from the Δ^5 to the Δ^4 position of 3-ketosteroids.

The oxidation of dehydroepiandrosterone (5-androsten-3β-ol-17-one) by bacterial β-hydroxysteroid dehydrogenase may be followed spectrophotometrically by the appearance of the absorption band of DPNH at 340 mμ[5]. Parallel measurements with crude enzyme preparations at 248 μm (λ max. for 4-androstene-3,17-dione in H_2O, ε = 16,300) showed a commensurate rate of formation of the conjugated double bond (appropriately compensated for the decrease in extinction at 248 mμ of DPN^+ upon reduction). 5-Androstene-3,17-dione (m.p. 157–158°)[6], a postulated intermediate in this reaction, readily undergoes acid and base catalyzed isomerization to 4-androstene-3,17-dione. Although quite stable at pH 7.0 and 25°, it is rapidly isomerized under these conditions by soluble extracts of *Pseudomonas* species grown on testosterone[4], by the supernatant of a rat liver homogenate centrifuged for 60 minutes at 100,000 *g*, and by human blood serum, but not by relatively large amounts of crystalline bovine serum albumin.

Unequivocal demonstration of the enzymic nature of this process and that it is distinct from the oxidizing enzyme, depend upon the following evidence: (1) The isomerizing activity is heat labile and is rapidly lost in dilute solutions even at 0° (50% of the activity was lost in 2 hours at 0° in solution containing 2.6 μg protein per ml). (2) The activity is precipitable from solution with ammonium sulfate and has been purified from bacterial extracts 20 to 30 fold by a four-step procedure (Table I). The last step has also been carried out chromatographically on calcium phosphate gel-celite columns utilizing gradient elution with increasing concentration of potassium phosphate buffer pH 7.0. This procedure has clearly separated β-hydroxysteroid dehydrogenase and isomerase peaks. (3) Isomerase is an adaptive enzyme and the specific activity in *Pseudomonas* extracts is enhanced by the presence of testosterone in the growth medium from about 7.7 units per mg protein to 300–600 units per mg protein. (4) The catalytic activities of the most purified preparations are extremely high, and the turnover numbers (assuming a mol wt. 100,000) are of the order of 130,000 per minute at 25°. (5) The isomerase activity varies with steroid concentration in accordance with the theory of MICHAELIS AND MENTEN[7]. The Michaelis constant for 5-androstene-3,17-dione at pH 7.0 and 25° was found to be $2.4 \cdot 10^{-5}$ *M*. (6) $5 \cdot 10^{-6}$ *M* $AgNO_3$ inactivates isomerase 50%, but unlike α- and β-hydroxysteroid dehydrogenases, isomerase is relatively insensitive to *p*-chloromercuribenzoate, and this constitutes an additional distinguishing feature from these enzymes. (7) Estradiol 3,17β is a powerful competitive inhibitor of bacterial isomerase.

TABLE I

PURIFICATION OF ISOMERASE ACTIVITY

Fraction	*Specific activity units/mg protein**
Initial extract	318
Step 1: 50–55% Ammonium sulfate fraction	575
Step 2: Protamine supernatant	—
Step 3: 40–45% Ammonium sulfate fraction	790
Step 4: Calcium phosphate gel supernatant	7230

* One unit of isomerase causes an initial optical density change of $\log_{10} I_0/I = 1.0$ per minute at 248 mμ and 25° in a 3.0 ml system (1.0 cm light path) containing 100 μM orthophosphate buffer pH 7.0 and 0.175 μM 5-androstene-3,17-dione in 0.1 ml CH_3OH.

In addition to 5-androstene-3,17-dione, the purified bacterial enzyme isomerized 5-pregnene-3,20-dione (m.p. 178–180°, $[\alpha]_D = +64.5°$)[8] to an α,β-unsaturated ketone. Another β,γ-unsaturated ketone, 5(10)-estren-17β-ol-3-one (a gift from Prof. A. J. BIRCH[9]), was converted to 4-estren-17β-ol-3-one (19 *nor*-testosterone). The Michaelis constant for 5(10)-estren-17β-ol-3-one was $2.8 \cdot 10^{-5}$ *M*, but the rate of its enzymic isomerization was only about 0.27% of that of 5-androstene-3,17-dione when both compounds were studied at a concentration of $6 \cdot 10^{-5}$ *M*. 5-cholesten-3-one

(a gift from Prof. L. F. FIESER[10]) was not a substrate for the bacterial isomerase. The isomerization reactions were found to be complete and reversibility could not be demonstrated.

The mechanism of the enzymic isomerization has been studied with the aid of D_2O. The enzymic reaction proceeds at identical rates in H_2O and 89% D_2O. Alkali catalyzed isomerization in D_2O results in the incorporation of deuterium from the medium, as first shown by ANCHEL AND SCHOENHEIMER[11] for the conversion of 5-cholesten-3-one to 4-cholesten-3-one. Three samples (2.5 mg) of 5-androstene-3,17-dione were isomerized at 25° respectively with 0.23 *N* HCl, 0.096 *N* NaOH and purified bacterial isomerase in a medium containing 89% D_2O. The reactions were complete in ten minutes or less; the products were isolated, chromatographed on silica gel by gradient elution, diluted with unlabeled 4-androstene-3,17-dione, repurified, and analyzed for deuterium in the mass spectrometer. Whereas the acid isomerized product contained 0.95 atoms D per molecule and the alkali isomerized material contained 3.86 atoms D per molecule, the enzyme isomerized product contained only 0.12 atoms D per molecule. A similar experiment with T_2O likewise revealed practically no incorporation of the tritium from the medium during the enzymic isomerization.

In the presence of alkali the two ketonic groups are enolized and this readily accounts for the incorporation of 4 deuterium atoms[12]. The enzymic mechanism suggests that there is a direct transfer of a proton from position 4 to 6 on the enzyme surface without exchange with the medium. If a direct intramolecular transfer of a proton does indeed occur, the steroid isomerase reaction provides the first recognized enzymic example. The direct and stereospecific nature of the transfer of hydrogen between substrate and coenzyme has been demonstrated in a number of enzymic reactions[13].

This investigation was supported in part by a grant from the American Cancer Society on recommendation of the Committee on Growth, National Research Council. The authors wish to thank Dr. B. VENNESLAND for valuable discussion, Dr. F. LOEWUS and Mr. L. GRAVES for the deuterium analyses and Drs. GEORGE T. OKITA and HAROLD WERBIN for the tritium analyses.

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Received July 15th, 1955

* Scholar of the American Cancer Society.

Effect of insulin on the metabolism of phosphorus in human erythrocytes*

KVAMME reported[1] that insulin, when injected *in vivo*, increased the turnover rate of ^{32}P in whole human blood; especially so in the 10 minute hydrolyzable fraction. GOURLEY[2], on the other hand, did not find any effect of insulin on either the distribution or on the turnover rate of any of the chemically separated phosphorus compounds of the erythrocytes of normal humans. In the

* Supported by the University of Illinois Foundation.