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Studies on the Stereospecificity of Liver Alcohol Dehydrogenase (LADH) for 3β-Hydroxy-5β-steroids. Inhibition Effect of Pyrazole and of a 3α-Hydroxycholanoic Acid*

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It was known from previous work that isoenzymes, "$LADH_S$", of liver alcohol dehydrogenase with NAD^+ can oxidize the 3β-hydroxy group of certain steroids, but not the corresponding 3α-hydroxy derivatives. The present work shows that a further requirement for activity is a 5β fusion between rings A and B. The reaction products were identified by gas chromatography and the reduction of NAD^+ followed fluorimetrically. 3α-Hydroxy-5β-cholanoic acid, pyrazole, 4-iodo-, 4-bromo-, and 4-methyl-pyrazole are all competitive inhibitors against 3β-hydroxy-5β-cholanoic acid, retinol, and ethanol, and the inhibition constants were determined.

In 1960, Ungar [1] observed that some 3β-hydroxy- but not 3α-hydroxysteroids can be oxidized to the corresponding 3-ketosteroids by horse liver alcohol dehydrogenase (horse LADH) with NAD^+ as coenzyme. The findings were confirmed by Ungar *et al.*,[2] Graves *et al.*,[3] and in this laboratory by Waller *et al.*[4] Pietruszko *et al.*[5] separated steroid active fractions from purely ethanol active fractions by gel electrophoresis of commercial LADH preparations, and Theorell *et al.*[6] soon afterwards crystallized an enzyme, "$LADH_S$", from the mother liquors of their own LADH preparations. $LADH_S$ was found to be active on both steroids and ethanol, whereas the steroid activity could be totally removed from the main fraction ($LADH_E$). The amino acid composi-

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tion and zinc content of $LADH_S$ were found to be, within the limits of error, the same as for $LADH_E$.

The studies mentioned above showed the specificity of what is here called $LADH_S$ for a β-configuration of the 3-hydroxy group. The present work demonstrates the importance of the configuration of the A/B ring fusion. When 5α- and 5β-isomers of androstane derivatives were tested as substrates it was found that horse LADH is stereospecific for 3β-hydroxy-5β-steroid derivatives. Rat LADH shows the same stereospecificity as horse LADH for 3β-hydroxy-5β-cholanoic acid (3β-OHCA).

The oxidation of the 3β-hydroxy group is inhibited by pyrazole which is a potent inhibitor of horse and rat LADH activity.[7,8] 3α-Hydroxy-5α-cholanoic acid, which is inactive as an LADH substrate and which is an inhibitor of the oxidation of 3β-hydroxy-5β-cholanoic acid catalyzed by horse LADH,[6] is also an inhibitor of the oxidation of ethanol, retinol and 3β-OHCA catalyzed by rat LADH.

MATERIALS AND METHODS

Crystalline horse LADH was prepared according to a modification of Dalziel's method.[9] To obtain a preparation containing as little ethanol as possible, the enzyme was dialyzed against 0.01 M phosphate buffer (pH 9.0) for two days.[10] This horse LADH preparation contained both $LADH_E$ and $LADH_S$. The activity of horse LADH was determined according to Dalziel [11] and its concentration was expressed as N, the normality of the coenzyme-binding capacity.

Rat LADH was prepared according to the method previously described. The activity is expressed as U: one unit is the amount of enzyme which catalyzes the transformation of 1 μmole/min of substrate under the conditions of the assay.

Chemicals. The 3-hydroxyandrostan-17-one isomers were purchased from Southestern Biochemicals, Inc., Morristown, Tennessee, USA, and Ikapharm, Ramat-Gan, Israel. The two 5α/β-androstane-3,17-dione isomers were obtained from Koch-Light Laboratory, Colnbrook, England. Dehydroepiandrosterone was kindly supplied by Dr. J. Babcock, Upjohn Company, Kalamazoo, Michigan, USA. The 3α- and 3β-hydroxy-5β-cholanoic acids (3α- and 3β-OHCA) were those used in our previous study.[4] NAD^+ and NADH were from Sigma Chemical Company, St. Louis, Missouri, USA. Retinol was obtained from Hoffmann-LaRoche, Basel. Pyrazole was purchased from Theodor Schuchardt, München. Pyrazole derivatives were gifts from Dr. B. Sjöberg, AB Astra, Södertälje, Sweden.

Solubilization of steroids. The neutral steroids were dispersed in aqueous solution with Tween 80 as described for retinol.[8,12,13] A mixture of acetone solutions of the steroid and Tween 80 were evaporated under nitrogen. The viscous residue was dispersed in the buffer. The optimal concentration of Tween 80 for solubilizing 100 μmole/l of steroid was about 0.05 % in the final solution (minimum 0.025 %). Above 0.1 %, inhibition of the enzymatic reaction occurs. Solutions of cholanoic acids (800 μM) were prepared by dissolving the compounds in 0.01 N NaOH.

Determination of enzymatic reaction rates with steroid substrates. The enzymatic reactions were carried out at 23.5°, in phosphate buffer (ionic strength 0.1, pH 7.0) and were followed by measurement of the fluorescence intensity of NADH.[14] The reaction was started by addition of the enzyme. With the horse LADH concentration used (0.95 μN), the aqueous Tween 80 solution showed a slight reaction with NAD^+ and NADH. The values of this reaction were subtracted from those obtained with Tween 80 suspensions of steroids. Studies of the inhibition of rat LADH by 3α-OHCA using ethanol and retinol as substrates were carried out according to the procedure previously described.[8]

Identification of end products by gas chromatography. In order to ascertain that the transformation of substrates occurred as well as the oxido-reduction of NAD(H), and to determine the nature of end products, experiments were performed in 20 ml reaction mixture. The steroids were then analyzed by gas chromatography. With 3β- and 3α-

hydroxy-5β-androstan-17-one and 3β-hydroxy-5α-androstan-17-one, the concentrations of the reagents were: 100 μM substrate, 500 μM NAD^+, 0.95 μN horse LADH. With 5α- and 5β-androstane-3,17-dione, the concentrations were: 100 μM substrate, 37 μM NADH, 0.95 μN LADH.

After an incubation period of 2 h, ten volumes of ethanol were added. The solution was filtered and taken to dryness *in vacuo*. The residue was partitioned between 20 ml of water and 50 ml of ethyl acetate. The water was extracted once more with ethyl acetate and the combined ethyl acetate phases were washed with aqueous sodium bicarbonate and then with water until neutral. After evaporation of the ethyl acetate the residue was analyzed by gas-liquid chromatography using a 2 m × 4 mm column of 3 % QF-1 on acid washed, silanized Gas-Chrom P.[4] The column temperature was about 205° and the argon inlet pressure about 1.5 kg/cm². Retention times were compared with those of appropriate reference compounds and peak areas were measured with a planimeter.

RESULTS

Stereospecificity of LADH with steroid substrates. As seen in Table 1 horse $LADH_S$ shows a high degree of specificity for 3β-hydroxy-5β-androstane derivatives. The gas chromatographic analyses show that 3β-hydroxy-5β-androstan-17-one is oxidized to 5β-androstane-3,17-dione and that the latter compound is reduced to 3β-hydroxy-5β-androstan-17-one. The 17-keto group is not reduced by the $LADH_S$ system. Furthermore, horse $LADH_S$ is essentially inactive with the 3-hydroxy-5α-androstan-17-one isomers.

Table 1. Substrate specificity of horse LADH: activity with androstane derivatives. The concentration of the substrates was 100 μM. The concentration of Tween 80 was 0.05 %. The concentrations of NAD^+ and NADH were 500 μM and 37—40 μM, respectively. LADH was a crystalline preparation containing both $LADH_E$ and $LADH_S$.

Substrates	Products determined by gas chromatography	Per cent conversion
3β-Hydroxy-5β-androstan-17-one	5β-androstane-3,17-dione	99
3α-Hydroxy-5β-androstan-17-one	5β-androstane-3,17-dione	0.4
5β-Androstane-3,17-dione	3β-hydroxy-5β-androstan-17-one	18
3β-Hydroxy-5α-androstan-17-one	5α-androstane-3,17-dione	0.9
3α-Hydroxy-5α-androstan-17-one		— [a]
5α-Androstane-3,17-dione	3α-hydroxy-5α-androstan-17-one	0.2
	3β-hydroxy-5α-androstan-17-one	0.5
3β-Hydroxyandrost-5-en-17-one		— [a]

[a] No NADH formed.

Rat LADH preparations catalyze the oxidation of 3β-hydroxy- but not that of 3α-hydroxy-5β-cholanoic acid. It is inactive with the 3-keto-Δ^4-steroids testosterone, progesterone, corticosterone, 11-deoxycorticosterone, and cortisol.

Kinetics of 3β-hydroxy-5β-cholanoic acid (3β-OHCA) oxidation catalyzed by rat LADH and pyrazole inhibition. In the 2—10 μM concentration range, the reaction rates follow a linear relation in the Lineweaver-Burk plots (Fig. 1). The Michaelis constant, K_m, is 2×10^{-6} M. The V_{max}/e is 17×10^{-3} μM U^{-1} l[1]

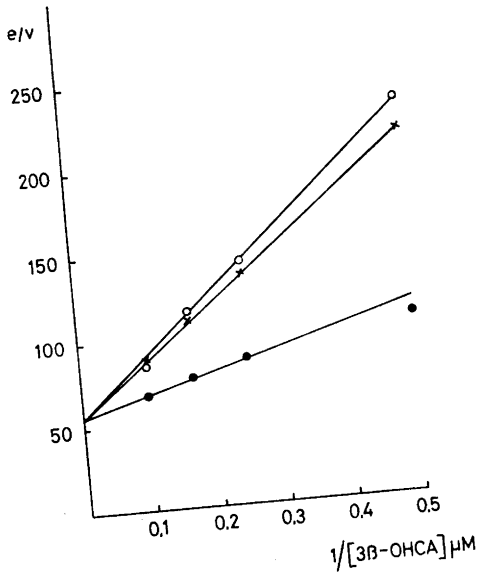

Fig. 1. Variations of the specific initial rate e/v at pH 7 and 23.5°C with the reciprocal of the 3β-hydroxy-5β-cholanoic acid concentration. Inhibition effect of 50 μM pyrazole concentration (○) and of 20 μM 3α-OHCA concentration (×). Control (●).

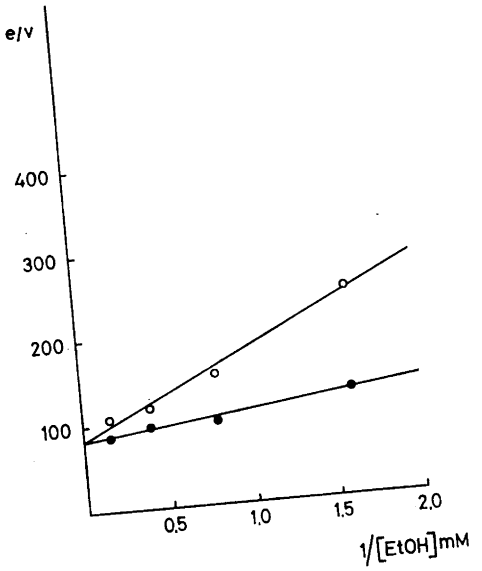

Fig. 2. Variations of the specific initial rate e/v pH 7 and 23.5°C with the reciprocal of the ethanol concentration (●). Inhibition effect of 8 μM 3α-OHCA concentration (○).

sec^{-1}. Pyrazole is a competitive inhibitor of 3β-OHCA oxidation (Fig. 1). The inhibition constant, K_i, is 25 μM. For 4-iodo-pyrazole, 4-bromo-pyrazole, and 4-methyl-pyrazole, the inhibition constants are 6, 9, and 10 μM, respectively (Table 2).

Table 2. Inhibitory effect of pyrazole derivatives on the oxidation of 3β-hydroxy-5β-cholanoic acid catalyzed by rat LADH.

	Pyrazole	4-Iodo-pyrazole	4-Bromo-pyrazole	4-Methyl-pyrazole
K_i (μM)	25	6	9	10

3α-Hydroxy-5β-cholanoic acid (3α-OHCA) inhibition effect. 3α-OHCA, which is not oxidized by rat LADH or horse LADH$_S$,[4] is an inhibitor of rat LADH activity. It is competitive with 3β-OHCA (Fig. 1), ethanol (Fig. 2), and retinol (Fig. 3) with the inhibition constants, K_i, 11, 2.9, and 2.5 μM, respectively (Table 3).

Table 3. Kinetic constants of rat ADH with various substrates and pyrazole and 3α-OHCA inhibition constants.

Substrates	Ethanol	Retinol	3β-OHCA
K_m (mM)	0.40	0.0008	0.002
V_{max}/e (μM U^{-1} l^{1} sec^{-1})	12×10^{-3}	1.7×10^{-3}	17×10^{-3}
K_i (μM) for 3α-OHCA	2.9	2.5	11
K_i (μM) for pyrazole	4.2	3.6	25

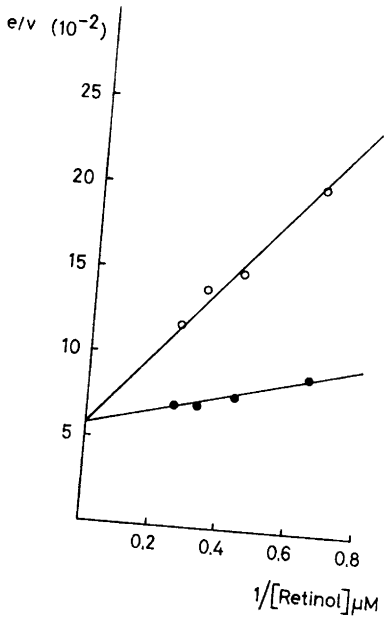

Fig. 3. Variations of the specific initial rate e/v at pH 7 and 23.5°C with the reciprocal of the retinol concentration (●). Inhibition effect of 8 μM 3α-OCHA concentration (○).

DISCUSSION

Previous authors have demonstrated the stereospecificity of LADH for 3β-hydroxy steroids.[1-5] The present work confirms these results and, moreover, shows the requirement for an A/B *cis*-fusion. The lack of activity towards a keto group at C-17 is in accordance with previous observations.[2,15]

Pyrazole is an inhibitor of rat LADH when the substrate is ethanol, retinol or 3β-OHCA. The inhibition constant with 3β-OHCA (25 μM) is higher

than with ethanol (4.2 μM) or retinol (3.6 μM). The difference in pyrazole inhibition between 3β-OHCA on one hand and retinol or ethanol on the other was explained by the crystallization of an enzyme, "$LADH_S$", from horse liver with different substrate binding sites for steroids and the other two alcohols.[6]

With the crude rat LADH preparations the comparison of the kinetic constants for 3β-OHCA oxidation with the kinetic constants of ethanol and retinol oxidation, shows that (a) the K_m value for 3β-OHCA is between the values observed for ethanol and retinol, and (b) the V_{max}/e is in the same range as that observed for ethanol and is higher than the value observed for retinol. In the case of horse $LADH_S$ and rat LADH, the values for K_m, with 3β-OHCA as substrate, are of the same order of magnitude as those reported by Talalay [16] for the NAD^+-linked bacterial 3β- and 17β-hydroxysteroid dehydrogenases.

The possible function of LADH in steroid metabolism is unknown. Among the naturally occurring steroid isomers, those with a 3β,5β configuration are quantitatively of little importance.[17] It could be that such steroids, if formed, are oxidized by $LADH_S$ and the resulting 3-keto grup subsequently reduced by a 3α-hydroxysteroid dehydrogenase. In preliminary studies it has been found that the rate of isotope elimination in the feces of rats given cholesterol-4-^{14}C is not changed by pyrazole inhibition of LADH.[18]

The inhibition by 3α-hydroxy-5β-cholanoic acid (lithocholic acid) of the oxidative reactions catalyzed by the rat LADH preparation is of interest. It very probably depends on the fact that the LADH preparation contains steroid active sites which react not only with steroid substrates but to a considerable extent also with ethanol. Lithocholic acid, which is formed by bacterial action on chenodeoxycholic (3α,7α-dihydroxy-5β-cholanoic) acid in the intestine,[19] produces liver cirrhosis in several animal species when given orally.[20] This effect, however, may not be directly related to the inhibitory effect on LADH since injections of lithocholic acid have also been shown to produce fever and local inflammatory reactions (see review by Kappas and Palmer [21]). Similar effects are seen with the stereochemically analogous 3α-hydroxy-5β-androstan-17-one.

On the other hand, the lithocholic acid inhibition effect makes possible an indirect relation between ADH activity and steroid metabolism. Since steroids with a 3α,5β configuration may control the ADH activity they may influence the metabolism of vitamin A, which is an essential cofactor in the adrenal steroid synthesis.[22–24] It is also known that some aldehyde dehydrogenases are steroid-sensitive.[25,26] Aldehyde dehydrogenase activity catalyzes the oxidation of the products of the ADH catalyzed reaction and thus can play a role in the vitamin A metabolism. The inhibition or activation of ADH and aldehyde dehydrogenase activities by steroids could be a way by which the control of the storage and distribution of vitamin A is performed in the mammalian organism.

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