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5β-CHOLESTANE-3α, 27-DIOL : NAD$^+$ OXIDOREDUCTASE AND 3α-HYDROXY-5β-CHOLESTAN-27-AL : NAD$^+$ OXIDOREDUCTASE IN RAT LIVER EXTRACT*)

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ABSTRACT

Using rat liver alcohol:NAD$^+$ oxidoreductase and aldehyde:NAD$^+$ oxidoreductase, the following facts were found:

1) 3α-hydroxy-5β-cholestan-27-al was reduced to 5β-cholestane-3α, 27-diol by liver alcohol:NAD$^+$ oxidoreductase.
2) The optimum pH was pH 7.0 with potassium phosphate buffer and pH 8.5 with Tris buffer.
3) The Km value of the substrate to the enzyme was 14.3 μM.
4) 3α-Hydroxy-5β-cholestan-27-al was oxidized to 3α-hydroxy-5β-cholestan-27-oic acid by liver aldehyde:NAD$^+$ oxidoreductase.
5) The optimum pH was pH 8.5 with Tris buffer.
6) The Km value of the substrate to the enzyme was 26.3 μM.

INTRODUCTION

Three pathways are presently proposed for the biosynthesis of lithocholic acid from cholesterol. The first one is that proposed by Samuelsson[1] who postulated that lithocholic acid is formed secondarily from chenodeoxycholic acid by the action of intestinal microorganisms. The second one is that proposed by Mitropoulos *et al*[2] who proposed that cholesterol is directly oxidized to give 3β-hydroxychol-5-en-24-oic acid, which is then converted to lithocholic acid. The third pathway is proposed by Kubota[3] in this laboratory and is essentially similar to that described for the biosynthesis of cholic acid. This pathway conforms to the postulate raised by Bergström and Lindstedt[4] that the modification of steroid nucleus preceds the degradation of the side chain. In this pathway 5β-cholestan-3α-ol, which has the same nucleus as lithocholic acid and the cholesterol-like side chain, is an intermediate. As a matter of fact Kubota has shown that 5β-cholestan-3α-ol is hydroxylated

*) 突合皐月：白ネズミ肝臓抽出液中の 5β-cholestane-3α, 27-diol : NAD$^+$ 酸化還元酵素ならびに 3α-hydroxy-5β-cholestan-27-al : NAD$^+$ 酸化還元酵素

at C_{27} to give 5β-cholestane-3α, 27-diol by rat liver mitochondria. Further metabolism of this sterol has, however, not been studied yet neither *in vivo* nor *in vitro*. Recently, the author described the partial synthesis of 5β-cholestane-3α, 27-diol and 3α-hydroxy-5β-cholestan-27-al, which are supposed to be intermediates in further metabolism of 5β-cholestane-3α, 27-diol. In this paper the oxidation of 5β-cholestane-3α, 27-diol to 3α-hydroxy-5β-cholestan-27-oic acid via 3α-hydroxy-5β-cholestan-27-al by rat liver extract is described.

MATERIALS AND METHODS

5β-Cholestane-3α, 27-diol, and 3α-hydroxy-5β-cholestan-27-al were synthesized according to the method described in the previous paper[5]. NAD^+, NADH (Grade III) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Sephadex G-100 was purchased from Pharmacia Chemicals (Uppsala, Sweden).

Liver alcohol:NAD^+ oxidoreductase (EC 1.1.1.1) was assayed as follows. 50 μl of 0.5M acetaldehyde was added to a cuvette (1-cm light path) containing 1.0 ml of 0.1 M phosphate buffer (pH 7.0), 50 μl of 5 mM NADH, enzyme solution and an appropriate amount of water to make a final volume of 3.0 ml. After rapid mixing, the decrease of absorption at 340 nm was measured using a spectrophotometer (JASCO Co., Tokyo, model UVIDEC 505) equipped with a recorder. The control cuvette contained the same components as sample cuvette except acetaldehyde. Aldehyde:NAD^+ oxidoreductase (EC 1.2.1.3) was assayed according to the following method. 100 μl of 0.5 M acetaldehyde was added to a cuvette (1-cm light path) containing 1.0 ml of 0.1 M pyrophosphate buffer (pH 9.3), 50 μl of 25 mM NAD^+, enzyme solution and an appropriate amount of water to make a final volume of 3.0 ml. After rapid mixing the increase of absorption at 340 nm was measured by the spectrophotometer. The control cuvette contained the same components as sample cuvette except acetaldehyde. 5β-Cholestane-3α, 27-diol:NAD^+ oxidoreductase was assayed according to the same method described for alcohol:NAD^+ oxidoreductase except using 3α-hydroxy-5β-cholestan-27-al as substrate, and 3α-hydroxy-5β-cholestan-27-al:NAD^+ oxidoreductase was assayed according to the same method described for aldehyde:NAD^+ oxidoreductase except using 3α-hydroxy-5β-cholestan-27-al as substrate.

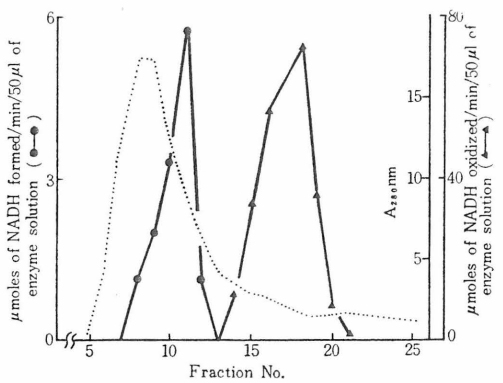


Fig. 1. Rat liver alcohol:NAD^+ oxidoreductase and aldehyde:NAD^+ oxidoreductase in the eluate of gel filtration on Sephadex G-100 of $(NH_4)_2SO_4$ preparation (0.2-0.6 satn) of rat liver extract. Assay methods are described in the text.
▲—▲ : alcohol:NAD^+ oxidoreductase, ●—● : aldehyde:NAD^+ oxidoreductase, ·····: protein concentration (A_{280nm})

Preparation of enzyme solution: The enzyme solutions were prepared according to the method described by Okuda *et al*[6]. 20 g of rat liver obtained from white male rats (Wistar strain) were homogenized in a Potter Elvehjem homogenizer with 40 ml of cold 0.1 M phosphate buffer (pH 7.0). The homogenate was allowed to stand for 1 h in the cold for extraction of enzyme, then centrifuged at 100,000 × g in a Hitachi preparative centrifuge (model 55P-2) for 1 h. The supernatant was fractionated with ammonium sulfate. The precipitate obtained from 0.2-0.6 ammonium sulfate saturation was dissolved in 0.35 M phosphate buffer (pH 7.8), and dialyzed against 0.007 M phosphate buffer (pH 7.8) for 20 h. 24 ml of the ammonium sulfate preparations were added to a Sephadex G-100 column (3.0 × 180 cm) and eluted with 2 l of 0.01 M phosphate buffer. Fractions of each 14.5 ml were collected at a rate of about 15 ml/h and each fraction was tested for alcohol:NAD^+ oxidoreductase and aldehyde:NAD^+ oxidoreductase activities. Fig. 1 shows these activities found in the effluents. In the following study the effluent in fraction 18 was used as alco-

hol:NAD$^+$ oxidoreductase and that from fraction 11 as aldehyde:NAD$^+$ oxidoreductase.

RESULTS

I. 5β-Cholestane-3α, 27-diol:NAD$^+$ Oxidoreductase

As described in the experimental section this enzyme activity was assayed by measuring its 3α-hydroxy-5β-cholestan-27-al reductase instead of 5β-cholestane-3α, 27-diol dehydrogenase activity.

Effect of pH on 3α-Hydroxy-5β-cholestan-27-al Reductase: To study the effect of pH on 3α-hydroxy-5β-cholestan-27-al reductase the substrate was incubated in different pH media with the enzyme (fraction 18 in Fig. 1) and NADH. As shown in Fig. 2, two peaks were observed in the region from pH 6.0–10.0, one was at pH 7.0 with potassium phosphate buffer and the enzyme activity was assayed only at this pH in the following study.

Effect of Time: The reduction of 3α-hydroxy-5β-cholestan-27-al was linear with time for only initial 2 min as shown in Fig. 3, after that the reaction velocity decreased fairly rapidly.

Effect of Enzyme Concentration: Fig. 4 shows the dependence of the reaction velocity on

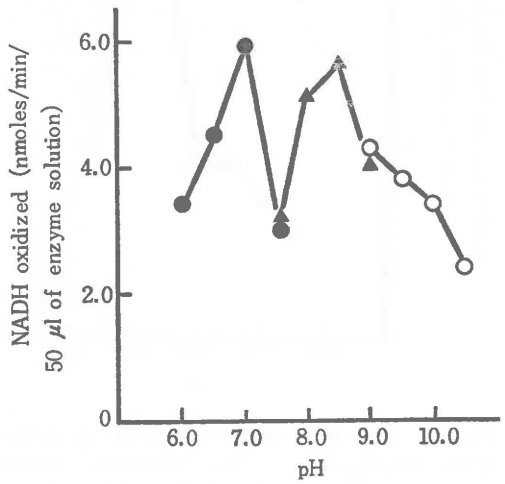


Fig. 2. Effect of pH on 3α-hydroxy-5β-cholestan-27-al reductase. The incubation mixture contained 0.12 μmole of 3α-hydroxy-5β-cholestan-27-al, 1.0 μmole of NADH, 50 μl of Fraction 18 (in Fig. 1) and 100 μmoles of potassium phosphate buffer (pH 6.0–7.5), 100 μmoles of Tris buffer (pH 7.5–9.0) or 100 μmoles of glycine buffer (pH 9.0–10.5) in a final volume of 3.0 ml. Incubations were conducted at 23°C.

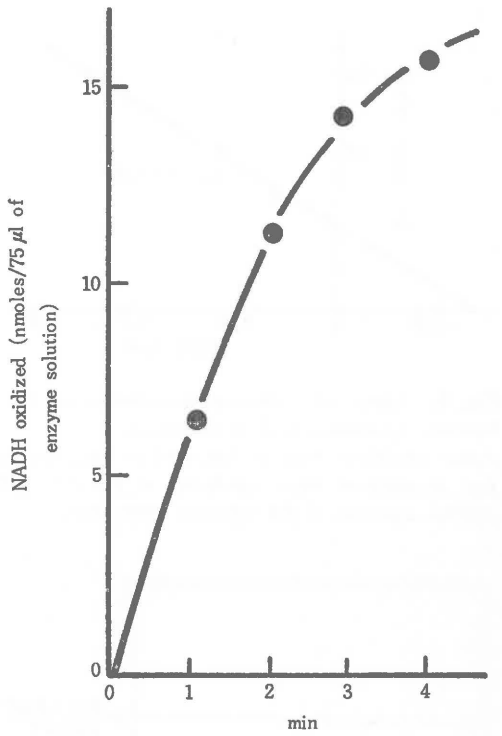


Fig. 3. Effect of time on 3α-hydroxy-5β-cholestan-27-al reductase. Assay conditions were as described in Fig. 2 except that incubations were conducted at pH 7.0 with potassium phosphate buffer.

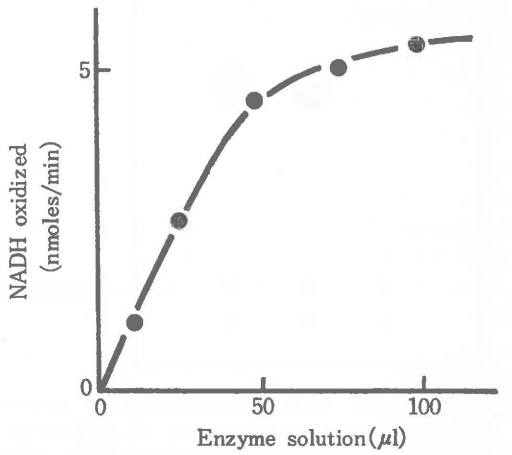


Fig. 4. Effect of enzyme concentration on 3α-hydroxy-5β-cholestan-27-al reductase.
Assay conditions were as described in Fig. 2 except that incubations were conducted at pH 7.0 and varying amounts fo enzyme fractions were used.

enzyme concentration. The linearity of the enzyme concentration-reaction velocity curve was seen in the range from 10–50 μl of enzyme solution, and the deviation from linearity was observed in higher enzyme concentration.

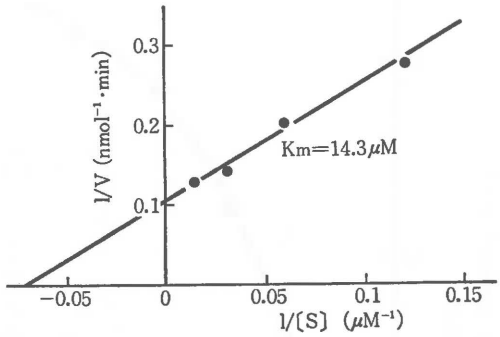


Fig. 5. Effect of substrate conentration on 3α-hydroxy-5β-cholestan-27-al reductase.
Assay conditions were as described in Fig. 2 except that incubations were conducted at pH 7.0 and varying amounts of the substrate were used.

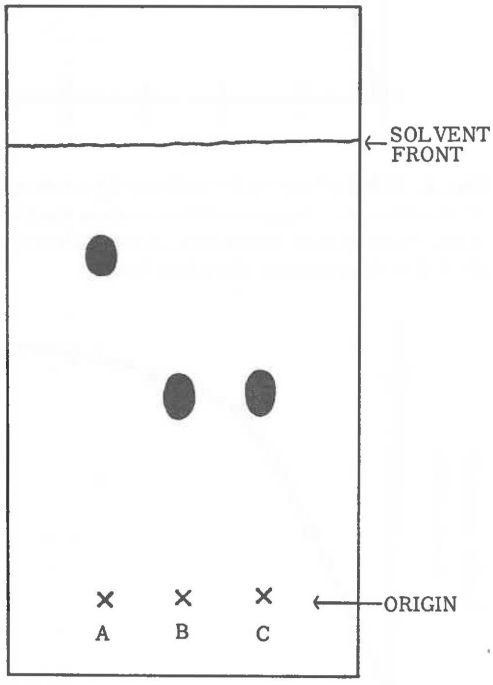


Fig. 6. Thin layer chromatogram of reaction products obtained by incubating 3α-hydroxy-5β-cholestan-27-al with rat liver alcohol:NAD$^+$ oxidoreductase and NADH.
Solvent system: benzene:ethyl acetate, 2 : 1
A : 3α-hydroxy-5β-cholestan-27-al,
B : reaction products
C : 5β-cholestane-3α, 27-diol

Effect of Substrate Concentration: The effect of substrate concentration on the reaction velocity was shown in Fig. 5 plotted according to the method of Lineweaver-Burk. The Michaelis constant (Km) of 3α-hydroxy-5β-cholestan-27-al was calculated to be 14.3 μM.

Identification of the Reaction Products: Fig. 6 shows a thin layer chromatogram of the products obtained by incubating 3α-hydroxy-5β-cholestan-27-al with the enzyme solution and NADH. As shown in the figure only one reaction product was obtained which ran at the same rate with 5β-cholestane-3α, 27-diol.

II. 3β-Hydroxy-5β-cholestan-27-al:NAD$^+$ Oxidoreductase

Effect of pH on 3α-Hydroxy-5β-cholestan-27-al:NAD$^+$ Oxidoreductase: Fig. 7 shows the dependence of 3α-hydroxy-5β-cholestan-27-al dehydrogenase activity on pH of media. As shown in the figure, the optimum pH was found at pH 8.5.

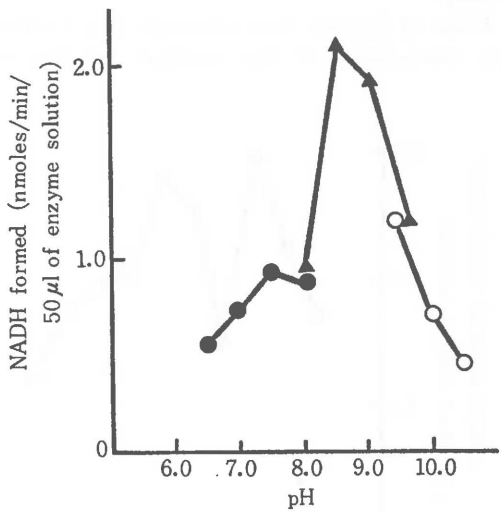


Fig. 7. Effect of pH on 3α-hydroxy-5β-cholestan-27-al dehydrogenase. The incubation mixture contained 0.12 μmole of 3α-hydroxy-5β-cholestan-27-al, 1.0 μmole of NAD$^+$, 50 μl of Fraction 11 (in Fig. 1) and 100 μmoles of potassium phosphate buffer (pH 6.0–7.5), 100 μmoles of Tris buffer (pH 7.5–9.0), or 100 μmoles of glycine buffer (pH 9.0–10.5) in a final volume of 3.0 ml. Incubations were conducted at 23°C.

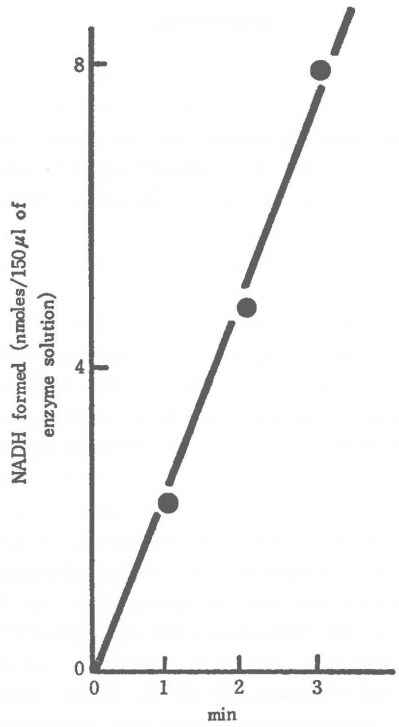


Fig. 8. Effect of time on 3α-hydroxy-5β-cholestan-27-al dehydrogenase.
Assay conditions were as described in Fig. 7 except that incubations were conducted at pH 8.5 with Tris buffer.

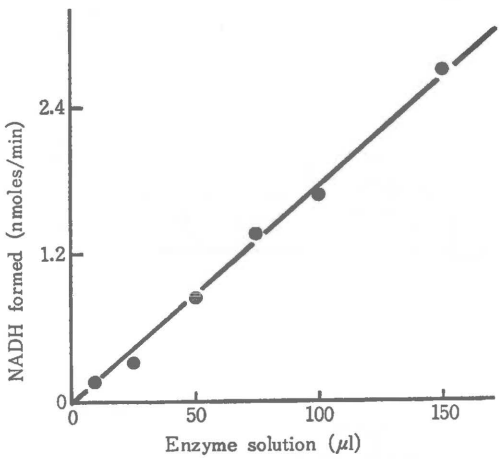


Fig. 9. Effect of enzyme concentractration on 3α-hydroxy-5β-cholestan-27-al dehydrogenase.
Assay conditions were as described in Fig. 7 except that incubations were conducted at pH 8.5 and varying amounts of enzyme solution were used.

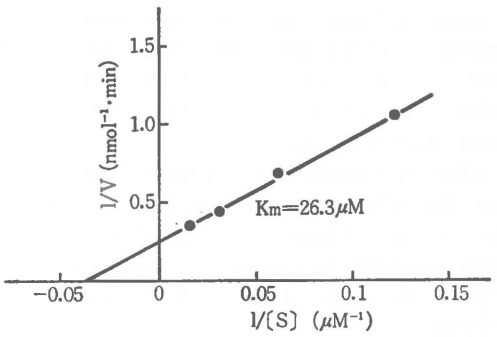


Fig. 10. Effecf of subdtrate concentration on 3α-hydroxy-5β-cholestan-27-al dehydrogenase.
Assay conditions were as described in Fig. 7 except that incubations were conducted at pH 8.5 and varying amounts of the substrate were used.

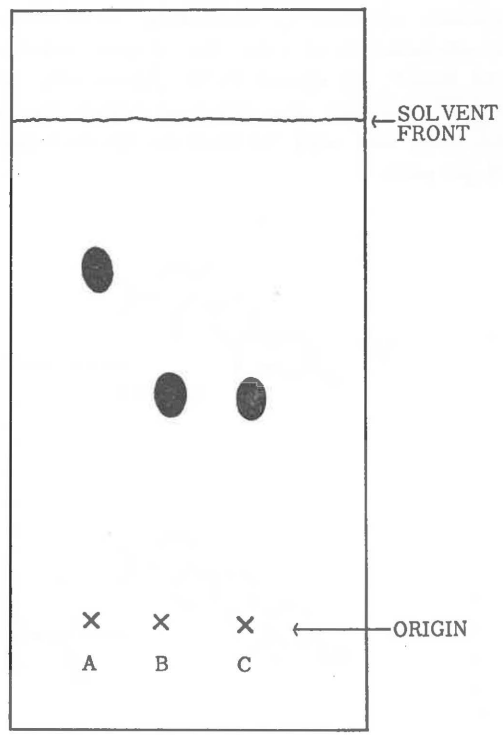


Fig. 11. Thin layer chromatogram of reaction products obtained by incubating 3α-hydroxy-5β-cholestan-27-al with rat liver aldehyde:NAD⁺ oxidoreductase and NAD⁺.
Solvent system: isooctane: ethyl acetate: acetic acid, 25 : 25 : 0.25
A : 3α-hydroxy-5β-cholestan-27-al,
B : reaction products
C : 3α-hydroxy-5β-cholestan-27-oic acid.

Effect of Time: The dehydrogenation of 3α-hydroxy-5β-cholestan-27-al was linear with time for at least initial 3 min which was adopted as standard incubation time for enzyme assay (Fig. 8).

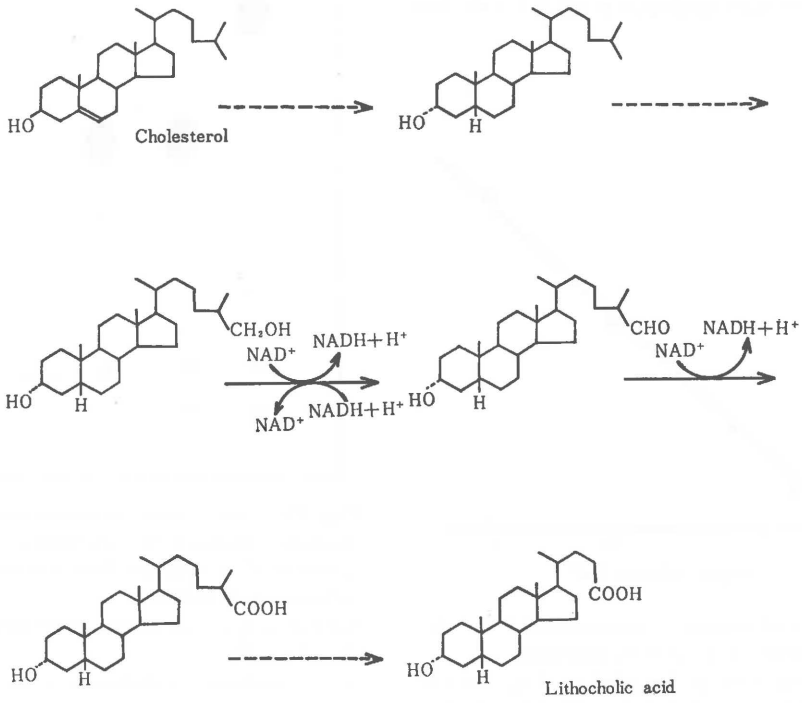
Effect of Enzyme Concentration: Fig. 9 shows the dependence of the reaction velocity on enzyme concentration. The linearity of the enzyme concentration-reaction velocity curve was seen in the range of 5–150 μl of the enzyme solution.

Effect of Substrate Concentration: The effect of substrate concentration on the reaction velocity was shown in Fig. 10 in double reciprocal plot. The Michaelis constant (Km) of 3α-hydroxy-5β-cholestan-27-al was calculated to be 26.3 μM.

Identification of Reaction Products: Fig. 11 shows a thin layer chromatogram of the products obtained by incubating 3α-hydroxy-5β-cholestan-27-al with the enzyme solution and NAD^+. As shown in the figure only one reaction product was obtained which ran at the same rate with 3α-hydroxy-5β-cholestan-27-oic acid.

DISCUSSION

It has been shown by Okuda *et al.*[7,8] that rat liver alcohol:NAD^+ oxidoreductase dehydrogenates 5β-cholestane-3α, 7α, 12α, 27-tetrol to 3α, 7α, 12α-trihydroxy-5β-cholestan-27-al, which is in turn oxidized by liver aldehyde:NAD^+ oxidoreductase to 3α, 7α, 12α-trihydroxy-5β-cholestan-27-oic acid, a precursor of cholic acid. The present results clearly demonstrate that rat liver alcohol:NAD^+ oxidoreductase catalyzes the dehydrogenation of 5β-cholestane-3α, 27-diol to 3α-hydroxy-5β-cholestan-27-al and rat liver aldehyde:NAD^+ oxidoreductase catalyzes the dehydrogenation of 3α-hydroxy-5β-cholestan-27-al to 3α-hydroxy-5β-cholestan-27-oic acid. Furthermore, Michaelis constant of 3α-hydroxy-5β-cholestan-27-al to liver alcohol:NAD^+ oxidoreductase was reasonably low and is comparable to that of 3α, 7α, 12α-trihydroxy-5β-cholestan-27-al (6 μM). Similarly, Michaelis constant of 3α-hydroxy-5β-cholestan-27-al to liver aldehyde:NAD^+ oxidoreductase was also comparable to that of 3α, 7α, 12α-trihydroxy-5β- cholestan-



27-al (22 μM). These results clearly show that 3α-hydroxy-5β-cholestan-27-al has as high affinity as 3α, 7α, 12α-trihydroxy-5β-cholestan-27-al to liver alcohol: NAD^+ oxidoreductase and liver aldehyde:NAD^+ oxidoreductase.

Since Kubota has shown that 5β-cholestan-3α-ol is hydroxylated at C_{27} in rat liver mitochondria, it may be suggested that 5β-cholestan-3α-ol is metabolized to 3α-hydroxy-5β-cholestan-27-oic acid by the action of liver alcohol:NAD^+ oxidoreductase and liver aldehyde:NAD^+ oxidoreductase in rat liver, which may be further metabolized to lithocholic acid by β-oxidation as shown in Diagram.

The present result also demonstrates that the presence of hydroxyl groups at C_7 and C_{12} in 5β-cholestane nucleus is not prerequisite for the action of both liver alcohol:NAD^+ oxidoreductase and liver aldehyde:NAD^+ oxidoreductase on the primary alcohol group and that of aldehyde of sterol, respectively.

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