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STEREOCHEMISTRY OF HYDROGEN TRANSFER BETWEEN PYRIDINE NUCLEOTIDE AND SOME INTERMEDIATES OF CHOLESTEROL CATABOLISM CATALYZED BY LIVER ALCOHOL AND ALDEHYDE DEHYDROGENASE

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SUMMARY

The stereochemistry of hydrogen transfer between pyridine nucleotide and some intermediates of cholesterol catabolism was studied.

1. The hydrogen transferred from NADH to 3α,7α,12α-trihydroxy-5β-cholestan-26-al, 3-oxo-5β-cholan-24-oic acid and to acetaldehyde catalyzed by rat liver alcohol:NAD oxidoreductase (EC 1.1.1.1) was the 4A-hydrogen.

2. The hydrogen transferred from 3α,7α,12α-trihydroxy-5β-cholestan-26-al and from acetaldehyde to NAD^+ catalyzed by horse liver aldehyde dehydrogenase (EC 1.2.1.3) was the 4A-hydrogen.

INTRODUCTION

It has been shown that the two hydrogen atoms at the 4 position of NADH are not situated identically, and dehydrogenases which transfer from NADH to a carbonyl group transfer either the 4A-hydrogen or the 4B-hydrogen [1]. This stereospecificity is usually the same whatever the source of the enzyme [2, 3].

We have been studying the dehydrogenases functioning in the cholesterol catabolism, and it was found that 5β-cholestane-3α,7α,12α,26-tetrol dehydrogenase is the same enzyme as liver alcohol:NAD oxidoreductase (liver alcohol dehydrogenase) [4] and 3α,7α,12α-trihydroxy-5β-cholestan-26-al dehydrogenase is the same enzyme as liver aldehyde:NAD oxidoreductase (liver aldehyde dehydrogenase) [5]. Recently, Hoshita [6] in this laboratory has shown that rat liver 3β-hydroxysteroid dehydrogenase is also the same enzyme as liver alcohol dehydrogenase. No studies on the stereochemistry of hydrogen transfer in these enzyme reactions have, however, been performed. The present experiments were attempted to establish the stereochemistry of hydrogen transfer between pyridine nucleotide and these steroids and to clarify the interrelationship of these dehydrogenases.

MATERIALS AND METHODS

Preparation of 3α,7α,12α-trihydroxy-5β-cholestan-26-al

3α,7α,12α-Trihydroxy-5β-cholestan-26-al was synthesized according to the method described by Okuda and Danielsson [7]. 3-Oxo-5β-cholan-24-oic acid was prepared by oxidation of 3α-hydroxy-5β-cholan-24-oic acid (lithocholic acid), and purified by aluminum oxide column chromatography. NAD, estradiol and β-hydroxysteroid dehydrogenase of *Pseudomonas testosteroni* were purchased from Sigma Chemical Co. (St. Louis, Mo.). Yeast alcohol dehydrogenase, and beef liver glutamic dehydrogenase were purchased from Boehringer Mannheim (Mannheim, Germany). QAE-Sephadex was purchased from Farmacia Fine Chemicals (Uppsala, Sweden). ^3H_2O was purchased from Radiochemical Centre (Amersham, England).

Preparation of [4-^{3}H]NAD$^+$

This was prepared from NAD$^+$, ^3H_2O and KCN according to the method described by Krakow et al. [8].

Preparation of [4A-^{3}H]NADH

This was prepared by incubating [4-^{3}H]NAD$^+$ with estradiol and β-hydroxysteroid dehydrogenase from *P. testosteroni*. After the completion of the reaction, the mixture was put on a column (4.3 cm × 25 cm) of QAE-Sephadex, which was equilibrated with 0.05 M Tris buffer (pH 8.8). The column was eluted with 0.1 M potassium phosphate, followed with 0.2 M potassium phosphate. Unchanged [4-^{3}H]NAD$^+$ was eluted at 0.1 M potassium phosphate concentration and [4A-^{3}H]-NADH was eluted at 0.2 M concentration as shown in Fig. 1. Fractions containing

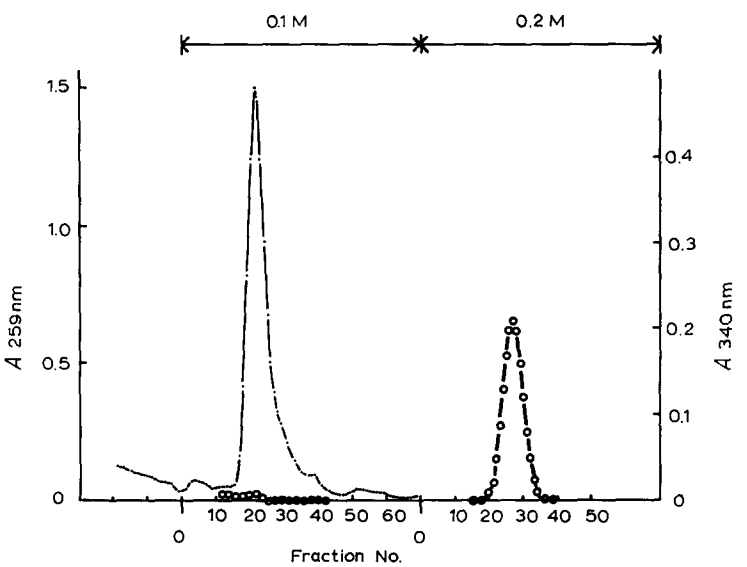

Fig. 1. QAE-Sephadex column chromatogram of pyridine nucleotide obtained by incubation of [4-^{3}H]NAD$^+$ with estradiol and β-hydroxysteroid dehydrogenase of *P. testosteroni*. —·—, $A_{259\ nm}$; —○—, $A_{340\ nm}$.

[4A-^{3}H]NADH were combined and concentrated by lyophilization. The specific activity of the isotope compound obtained was $10.9 \cdot 10^5$ dpm/μmole.

Preparation of [4B-^{3}H]NADH

This was prepared by incubating [4-^{3}H]NAD$^+$ with ethanol and yeast alcohol dehydrogenase. The reaction mixture was treated in the same way as described above, and [4B-^{3}H]NADH was separated from unchanged [4-^{3}H]NAD$^+$ and concentrated by lyophilization. The specific activity of the isotope compound obtained was $8.8 \cdot 10^5$ dpm/μmole.

Enzyme preparations

Rat liver alcohol dehydrogenase was prepared according to the method described in the previous paper of this laboratory [4]. The enzyme solution used was the material obtained after hydroxylapatite column chromatography (0.6 mg of protein/ml). Horse liver aldehyde dehydrogenase was prepared according to the method described by Okuda et al. [5], and the enzyme solution was the material obtained after hydroxylapatite column chromatography (0.3 mg of protein/ml).

Incubations

Incubations with 3α,7α,12α-trihydroxy-5β-cholestan-26-al. 0.5 μmole of [4A-^{3}H]-NADH, or [4B-^{3}H]NADH was dissolved in a small amount of water and pH of the solution was adjusted to 8.0 by adding phosphoric acid. To this solution were added 0.12 μmole of 3α,7α,12α-trihydroxy-5β-cholestan-26-al and 3.0 ml of rat liver alcohol dehydrogenase. Incubations were conducted for 2 h at 37 °C. The reaction mixture was heated on a boiling water bath for 2 min, cooled and centrifuged. The pH of the supernatant was adjusted to 8.8 with 2 M NaOH. Then the solution was put on a column of QAE-Sephadex, equilibrated with 0.05 M Tris buffer (pH 8.8). The column was eluted with 0.1 M potassium phosphate. Each 2.3 ml was collected and aliquots were used to measure both radioactivity and absorbance at 259 nm. Fractions containing NAD$^+$ were combined. To this solution were added 50 mg of diluent nicotinamide and 2 M NaOH to make the pH of the solution 9.6. The mixture was placed in a boiling water bath to hydrolyze NAD$^+$. After readjustment of the pH to 7.8 with dilute H_2SO_4, the sample was extracted with diethyl ether [8]. The solvent was removed by evaporation, and the nicotinamide was recrystallized from benzene, m.p. 130–131 °C. Two samples were weighed and counted in a liquid scintillation counter.

Incubations with 3-oxo-5β-cholan-24-oic acid. 0.5 μmole of [4A-^{3}H]NADH, or [4B-^{3}H]NADH was dissolved in a small amount of water and the pH of the solution was adjusted to 6.5 as described above. To this solution were added 0.25 μmole of sodium 3-oxo-5β-cholan-24-oate and 3.0 ml of rat liver alcohol dehydrogenase. Incubations were conducted for 3.5 h at 37 °C. The reaction mixture was treated in the same way as described above.

Incubations with 3α,7α,12α-trihydroxy-5β-cholestan-26-al with horse liver aldehyde dehydrogenase. To 39 ml of horse liver aldehyde dehydrogenase were added 25 μmoles of [4-^{3}H]NAD$^+$, 21 ml of pyrophosphate buffer (pH 9.3) and 5.0 μmoles of 3α,7α,12α-trihydroxy-5β-cholestan-26-al. Incubations were conducted for 2.5 h at 37 °C. The reaction mixture was subjected to QAE-Sephadex column chromatography

as described above. Fractions corresponding to NADH were combined and concentrated by lyophilization.

Incubations of reduced pyridine nucleotide, obtained by incubations of [4-^{3}H]-NAD$^+$ with horse liver aldehyde dehyrdogenase and 3α,7α,12α-trihydroxy-5β-cholestan-26-al, with the enzymes of known specificities. Incubations with yeast alcohol dehydrogenase, which is known to transfer the 4A-hydrogen, were performed according to the usual method, and the reaction mixture was treated as described above. Incubations with beef liver glutamic dehydrogenase, which transfers the 4B-hydrogen, were performed according to the method described by Krakow et al. [8], and the reaction products were subjected to the same procedure as described above.

Radioactivity was measured by a liquid scintillation counter (Aloka Co., Tokyo, Model LSC-601). Radioactive compounds were dissolved in 10 ml of a solution consisting of 4 g of 2,5-diphenyloxazole, 100 mg of 2,2′-*p*-phenylene-bis-(5-phenylozaxole), 700 ml of toluene and 300 ml of ethanol. With the settings used the counting efficiency was 30% for tritium.

RESULTS

Incubations of 3α,7α,12α-trihydroxy-5β-cholestan-26-al with rat liver alcohol dehydrogenase

The effluents corresponding to NAD$^+$ from the QAE-Sephadex column, on

TABLE I

SUCCESSIVE CRYSTALLIZATION OF NICOTINAMIDE OBTAINED BY ALKALINE HYDROLYSIS OF [4-^{3}H]NAD$^+$ PRODUCED BY INCUBATION WITH RAT LIVER ALCOHOL DEHYDROGENASE

Reactions	No.	Wt (mg)	Total activity (dpm)	Spec. act. (dpm/mg)
3α,7α,12α-Trihydroxy-5β-cholestan-26-al + [4A-^{3}H]NADH + H$^+$ → 5β-cholestane-3α,7α,12α,26-tetrol + NAD$^+$	1	12.6	790	63
	2	9.7	424	44
	3	5.2	252	48
3α,7α,12α-Trihydroxy-5β-cholestan-26-al + [4B-^{3}H]NADH + H$^+$ → 5β-cholestane-3α,7α,12α,26-tetrol + NAD$^+$	1	12.1	7 280	602
	2	9.6	5 453	568
	3	6.7	3 772	563
3-Oxo-5β-cholan-24-oic acid + [4A-^{3}H]NADH + H$^+$ → 3β-hydroxy-5β-cholan-24-oic acid + NAD$^+$	1	4.8	570	119
	2	3.6	387	107
	3	3.1	287	92
3-Oxo-5β-cholan-24-oic acid + [4B-^{3}H]NADH + H$^+$ → 3β-hydroxy-5β-cholan-24-oic acid + NAD$^+$	1	15.6	8 990	576
	2	12.3	6 232	507
	3	10.8	5 484	507
Acetaldehyde + [4A-^{3}H]NADH + H$^+$ → ethanol + NAD$^+$	1	10.2	540	50
	2	7.6	376	49
	3	6.7	366	55
Acetaldehyde + [4B-^{3}H]NADH + H$^+$ → ethanol + NAD$^+$	1	12.8	12 190	952
	2	10.4	9 296	894
	3	8.7	7 673	882

which the reaction mixture obtained by incubation with [4A-^{3}H]NADH or [4B-^{3}H]NADH was applied, were combined and treated as described in the previous section. The nicotinamide obtained by hydrolysis of NAD^+ was recrystallized to a constant specific activity (Table I). The results are summarized in Table II. 100% of the original isotope was retained in NAD^+ obtained after incubation of [4B-^{3}H]-NADH, whereas almost 90% of the original isotope was lost in NAD^+ obtained after incubation of [4A-^{3}H]NADH, demonstrating that in the hydrogen transfer from NADH to 3α,7α,12α-trihydroxy-5β-cholestan-26-al the 4A-hydrogen is specifically transferred.

TABLE II

HYDROGEN TRANSFER FROM [4A-^{3}H]NADH AND [4B-^{3}H]NADH IN REACTIONS CATALYZED BY RAT LIVER ALCOHOL DEHYDROGENASE

Substrates	Coenzymes	Spec. act. of NADH used (dpm/μmole)	Spec. act. of NAD^+ produced (dpm/μmole)	Tritium retained (%)
3α,7α,12α-Trihydroxy-5β-cholestan-26-al	[4A-^{3}H]NADH	$10.9 \cdot 10^5$	$1.5 \cdot 10^5$	13
	[4B-^{3}H]NADH	$8.8 \cdot 10^5$	$9.2 \cdot 10^5$	104
3-Oxo-5β-cholan-24-oic acid	[4A-^{3}H]NADH	$10.9 \cdot 10^5$	$1.6 \cdot 10^5$	14
	[4B-^{3}H]NADH	$8.8 \cdot 10^5$	$8.1 \cdot 10^5$	91
Acetaldehyde	[4A-^{3}H]NADH	$10.9 \cdot 10^5$	$1.1 \cdot 10^5$	10
	[4B-^{3}H]NADH	$8.8 \cdot 10^5$	$8.9 \cdot 10^5$	101

Incubations of 3-oxo-5β-cholan-24-oic acid with rat liver alcohol dehydrogenase

The results are summarized in Tables I and II. It is apparent from the tables that the hydrogen transferred in this case is the 4A-hydrogen.

Incubations of acetaldehyde with rat liver alcohol dehydrogenase and [4A-^{3}H]NADH or [4B-^{3}H]NADH

These incubations were also performed in the same conditions applied at the present experiments for comparison, and the results are summarized in Tables I and II, which results are consistent with those obtained with horse liver alcohol dehydrogenase [9].

Incubations of 3α,7α,12α-trihydroxy-5β-cholestan-26-al with horse liver aldehyde dehydrogenase

3α,7α,12α-Trihydroxy-5β-cholestan-26-al dehydrogenase does not catalyze the reverse reaction practically. It was, therefore, necessary to incubate aldehyde with the oxidized form of pyridine nucleotide first, and the reduced coenzyme obtained should be reoxidized by the enzymes whose stereospecificities are known. Thus, the steroid aldehyde was incubated with horse liver aldehyde dehydrogenase first, and the reduced pyridine nucleotide was isolated by the QAE-Sephadex column chromatography. The reason why the reduced pyridine nucleotide should have been isolated by chromatography will be discussed in the following section. The reduced pyridine

TABLE III

SUCCESSIVE CRYSTALLIZATION OF NICOTINAMIDE OBTAINED BY ALKALINE HYDROLYSIS OF [4-^{3}H]NAD$^+$ PRODUCED BY THE 2ND INCUBATION OF NADH, WHICH WAS PREPARED BY INCUBATION WITH HORSE LIVER ALDEHYDE DEHYDROGENASE

First incubations	Enzymes and substrates in 2nd incubation	No.	Wt (mg)	Total activity (dpm)	Spec. act. (dpm/mg)
3α,7α,12α-Trihydroxy-5β-cholestan-26-al + [4-^{3}H]NAD$^+$ + H_2O→ 3α,7α,-12α-trihydroxy-5β-cholestan-26-oic acid + NADH + H$^+$	Alcohol dehydrogenase and acetaldehyde	1	14.3	7 100	496
		2	12.0	5 112	426
		3	9.2	3 090	336
	Glutamic dehydrogenase and α-ketoglutarate	1	7.1	1 712	241
		2	5.9	1 038	176
		3	3.4	582	171
Acetaldehyde + [4-^{3}H]NAD$^+$ + H_2O→ acetic acid + NADH + H$^+$	Alcohol dehydrogenase and acetaldehyde	1	12.7	12 100	953
		2	9.0	8 464	940
		3	7.2	5 238	728
	Glutamic dehydrogenase and α-ketoglutarate	1	7.5	2 630	350
		2	2.3	780	339
		3	1.3	408	313

nucleotide thus obtained was then incubated with the enzymes of known specificities and treated in the same way as described. The results are summarized in Tables III and IV. [4-^{3}H]NADH obtained by incubating 3α,7α,12α-trihydroxy-5β-cholestan-26-al did not lose its radioactivity by incubation with yeast alcohol dehydrogenase and acetaldehyde, whereas it was lost significantly by incubation with glutamic dehydrogenase of beef liver and α-ketoglutarate, demonstrating that the hydrogen transferred from 3α,7α,12α-trihydroxy-5β-cholestan-26-al to NAD$^+$ is stereospecifically to the 4A-position.

TABLE IV

HYDROGEN TRANSFER FROM 3α,7α,12α-TRIHYDROXY-5β-CHOLESTAN-26-AL AND ACETALDEHYDE CATALYZED BY HORSE LIVER ALDEHYDE DEHYDROGENASE

Substrates	Enzymes and substrates in the 2nd incubation	Spec. act. of NADH produced in the 1st incubation (dpm/μmole)	Spec. act. of NAD$^+$ produced in the 2nd incubation (dpm/μmole)	Tritium retained after 2nd incubation (%)
3α,7α,12α-Trihydroxy-5β-cholestan-26-al	Alcohol dehydrogenase and acetaldehyde	$10.6 \cdot 10^5$	$9.0 \cdot 10^5$	85
	Glutamic dehydrogenase and α-ketoglutarate	$10.6 \cdot 10^5$	$2.4 \cdot 10^5$	23
Acetaldehyde	Alcohol dehydrogenase and acetaldehyde	$9.6 \cdot 10^5$	$8.9 \cdot 10^5$	93
	Glutamic dehydrogenase and α-ketoglutarate	$9.6 \cdot 10^5$	$2.0 \cdot 10^5$	20

Incubations of acetaldehyde with horse liver aldehyde dehydrogenase

The incubations were also performed in the same conditions, and the results are summarized in Tables III and IV, which are similar to those obtained with beef liver aldehyde dehydrogenase [9].

DISCUSSION

Methods

One of the difficulties in the study of the stereochemistry of hydrogen in steroid dehydrogenase is that the conversion of coenzyme by these enzymes can not be complete as shown in Fig. 1, because the solubility of the steroids is too low to make the incubation mixture saturated with the steroid, while we have to saturate the mixture with the coenzyme to attain the maximum velocity and to obtain as much oxidized or reduced coenzyme as possible in a limited time of incubation. If the amount of unchanged coenzyme is significant, the reaction mixture cannot be subjected to the second incubation with the enzymes of known specificities and to the following alkaline hydrolysis as Krakow et al. [8] have done. If it were done, the coenzyme obtained by the second incubation could not be differentiated from the original one leading to an equivocal conclusion. That is because in the present experiment the reaction mixtures were subjected to QAE-Sephadex column chromatography after each enzyme reaction, which was found to be efficient to separate NAD^+ from NADH. The present method will also be applicable in the study of the stereochemistry of the hydrogen from low solubility compounds.

3β-Hydroxysteroid dehydrogenase

As shown in the previous section the hydrogen transferred from NADH to 3-oxo-5β-cholanoate was found to be 4A-specific. This does not conform with the general concept that the stereospecificity of dehydrogenase is usually the same whatever the source of enzyme, because 3β-hydroxysteroid dehydrogenase of *P. testosteroni* transfers the 4B-hydrogen [10]. Similar discrepancies are also found in other cases. Thus alcohol dehydrogenase from kidney transfers the 4B-hydrogen [11], whereas alcohol dehydrogenase from liver transfers the 4A-hydrogen [9], and 3α-hydroxysteroid dehydrogenase of *P. testosteroni* transfers the 4B-hydrogen [10] but that of the rat liver used the 4A-hydrogen [12]. The above mentioned generalization therefore, may not hold true at least with these enzymes.

Liver alcohol dehydrogenase and liver aldehyde dehydrogenase

The fact that the hydrogen transfers from NADH to 3α,7α,12α-trihydroxy-5β-cholestan-26-al, or to 3-oxo-5β-cholan-24-oic acid showed the same specificity as that from NADH to acetaldehyde may be consistent with the assumption that all three enzyme activities are catalyzed by the common catalytic center(s), although it may not exclude the possibility that each enzyme activity is due to each different center(s) of the same stereospecificity.

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