Separation of 5β-Cholestane-3α,7α,12α,26-tetrol Oxidoreductase, Ethanol-NAD Oxidoreductase, 5β-Cholestane-3α,7α,12α-triol-26-al Oxidoreductase, and Acetaldehyde-NAD Oxidoreductase from the Soluble Fraction of Rat Liver by Gel Filtration

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In the previous report (Okuda et al., 1968) it was suggested that TeHC, which is an intermediate in the conversion of cholesterol to cholic acid (Danielsson, 1963), is dehydrogenated to THCA by two NAD-linked enzymes, namely TeHC-NAD oxidoreductase and THAL-NAD oxidoreductase in rat liver soluble fraction. In this communication, it will be reported that the TeHC-NAD oxidoreductase and THAL-NAD oxidoreductase from the soluble fraction of rat liver are distinct and separate enzymes, furthermore it will be suggested that TeHC-NAD oxidoreductase activity and ethanol-NAD oxidoreductase (EC 1.1.1.1) activity are due to the same enzyme, and THAL-NAD oxidoreductase activity and acetaldehyde-NAD oxidoreductase (EC 1.2.1.3) activity are due to the same enzyme.

Materials and Methods

Tritium labeled TeHC and THAL were synthesized as described before (Okuda et al., 1965; Okuda et al., 1968). NAD was purchased from

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Abbreviations: TeHC, 5β-cholestane-3α,7α,12α,26-tetrol; THAL, 5β-cholestane-3α,7α,12α-triol-26-al; THCA, 5β-cholestane-3α,7α,12α-triol-26-oic acid

Sigma Chemical Co. (St. Louis Mo. U.S.A.). Sephadex G-100 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Ethanol-NAD oxidoreductase activity was assayed according to the procedure described by Dalziel (1957). Acetaldehyde-NAD oxidoreductase activity was assayed according to the method described by Racker (1955). Hitachi spectrophotometer Model 124 was used for measurement of absorbancy at 340 mμ. TeHC-NAD oxidoreductase activity was assayed according to the method standardized by us (Okuda et al., 1968), and the activity for reverse reaction was assayed as follows: The substrate, tritium labeled THAL (2.8×10^8 d.p.m. per mg) was dissolved in methanol and the specified amount (25 μg in 10 μl) was pipetted into individual 25 ml test tube. To this solution 0.5 ml of 0.1M phosphate buffer, pH 7.4, and 0.05 ml of 0.025M NADH were added. The total volume was brought to 1.5 ml by addition of appropriate amount of water. The mixture was then shaken in a bath, maintained at 37°, for a few minutes to equilibrate the temperature, and the incubation was started by addition of 25 μl of enzyme solution into this mixture. Incubations were conducted for 5 minutes at 37° with constant mechanical agitation. After 5 minutes the tubes were placed in crushed ice to cool the mixtures and 2 drops of 2N HCl were added in 10 seconds, then the solution was mixed with 5 ml of ethyl acetate and the whole mixture was agitated with mechanical agitator. Aliquot (4 ml) of ethyl acetate extract was taken into another tube and washed with water, and the solvent was evaporated by blowing nitrogen gas. The residue was analyzed according to the method described in the previous paper (Okuda et al., 1968). Aloka radiochromatogram scanner Model TLC-1 was used for scanning the thin layer plates.

THAL-NAD oxidoreductase activity was determined essentially in the same way as described above except using NAD as acceptor and 0.1M phosphate buffer, pH 8.0, as buffer solution.

Fractionation procedure: From white male rats (Wistar strain) rat liver

fractions were obtained. Twenty g of tissue were homogenized in a Potter-Elvehjem homogenizer with 40 ml of cold 0.1M phosphate buffer, pH 7.0. The homogenate was centrifuged at 77,000xg in a Hitachi preparative ultracentrifuge Model 55P-2 for 1 hr.

The supernatant was fractionated with ammonium sulfate. The precipitate obtained from the 0.3-0.6 ammonium sulfate saturation was dissolved in 0.035M phosphate buffer, pH 7.8, and dialyzed against 0.007M phosphate buffer, pH 7.8, for 20 hr. Twenty seven ml of the ammonium sulfate preparations were added to the Sephadex column (3.0x140 cm) and eluted with 2 liters of 0.01M phosphate buffer, pH 7.8. Fractions of 17.5 ml were collected at a rate of about 15 ml/hr and tested for TeHC-NAD oxidoreductase, THAL-NAD oxidoreductase, ethanol-NAD oxidoreductase and acetaldehyde-NAD oxidoreductase activities.

Results and Discussion

As previously reported, one mole of TeHC was dehydrogenated to THCA with concomitant reduction of two moles of NAD in rat liver soluble fraction, and this was also the case with the enzymes precipitated from the 0.3-0.6 ammonium sulfate saturation (Table I). However, when the enzymes were subjected to Sephadex G-100, the TeHC dehydrogenase activity was hardly detected in any fraction of the eluate. It seemed at first as if the enzyme were deteriorated. But this was not the case. When TeHC-NAD oxidoreductase was assayed by measuring reverse reaction, strong activity was observed (Table I, Fig. 1). After gel filtration step, therefore, the TeHC-NAD oxidoreductase was assayed by measuring reverse reaction.

The separation of the TeHC-NAD oxidoreductase activity, THAL-NAD oxidoreductase activity, ethanol-NAD oxidoreductase activity and acetaldehyde-NAD oxidoreductase activity of the fraction obtained from ammonium sulfate fraction of the soluble fraction of rat liver is illustrated in Fig. 1. In Fig. 1, 27 ml of the ammonium sulfate fraction (0.3-0.6

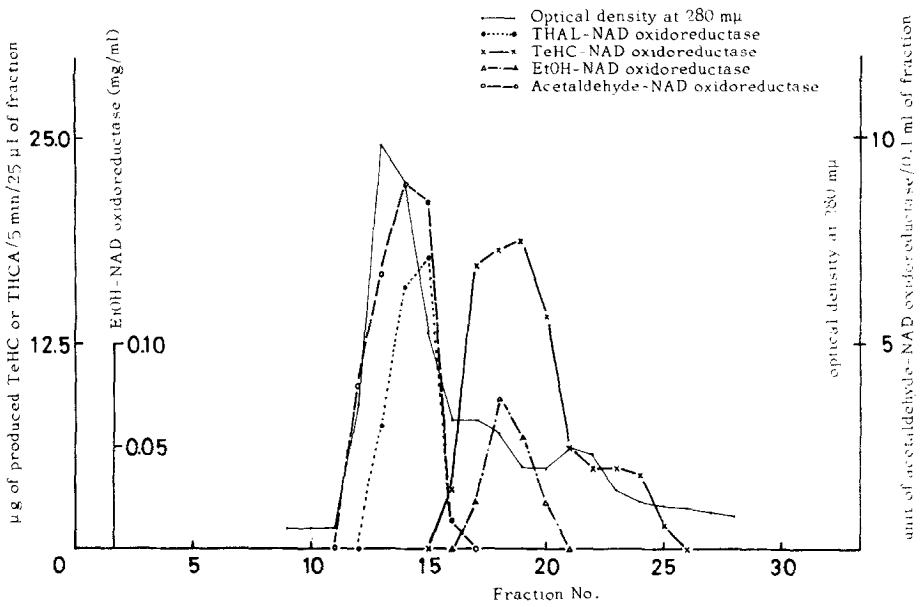

Fig. 1. TeHC-NAD oxidoreductase, EtOH-NAD oxidoreductase, THAL-NAD oxidoreductase and acetaldehyde-NAD oxidoreductase activities in gel filtration of rat liver.

saturation) dissolved in 0.035M phosphate buffer, pH 7.8, containing 1110 mg of protein were placed on the Sephadex column. Elution of the protein was carried out with 0.01M phosphate buffer, pH 7.8. Fractions of 17.5 ml were collected at a rate of 15 ml/hr. Protein content of the fractions was estimated by measurement of absorbancy at 280 mμ. The TeHC-NAD oxidoreductase, THAL-NAD oxidoreductase, ethanol-NAD oxidoreductase and acetaldehyde-NAD oxidoreductase activities were assayed in systems containing 25 μl, 25 μl, 0.1 ml and 0.1 ml of fractions respectively. The assays were performed as described above. From the results of this study, it can be concluded that the TeHC-NAD oxidoreductase and THAL-NAD oxidoreductase from the soluble fraction of rat liver are distinct and separate enzymes.

As mentioned above, if the eluate from Sephadex column was tested

for TeHC dehydrogenase activity using TeHC and NAD as substrates, no appreciable activity was observed in any fraction of the eluate. However, when 25 µl of fraction No. 14 (where the THAL-NAD oxidoreductase activity was maximum) and 25 µl of fraction No. 19 (where the TeHC-NAD oxidoreductase activity was maximum) were mixed and incubated with TeHC and NAD, a marked dehydrogenation activity of TeHC to THCA was observed as shown in Table I. These results seem to suggest that the dehydrogenation of TeHC to THCA is an enzyme catalyzed coupled reaction, where an exergonic

Table I

TeHC- and THAL-NAD Oxidoreductase Activities in Different Fractions of Rat Liver

Fractions	Substrates	Coenzymes	Products (%) TeHC	THAL	THCA
77,000xg supernatant	TeHC	NAD	17.3	0	82.7
Ammonium sulfate fraction (0.3-0.6 saturation)	TeHC	NAD	22.7	0	77.3
Gel filtration Fraction No. 14	TeHC	NAD	100.0	0	0
	THAL	NADH	0	100.0	0
	THAL	NAD	0	18.2	81.8
Fraction No. 19	TeHC	NAD	100.0	0	0
	THAL	NADH	73.6	26.4	0
Fraction No. 14 + fraction No. 19	TeHC	NAD	59.3	0	32.8

process (the dehydrogenation of THAL to THCA) may be coupled with the endergonic conversion of TeHC to THAL.

The fact that the TeHC-NAD oxidoreductase activity curve coincides with that of ethanol-NAD oxidoreductase (Fig. 1) seems to suggest that both enzyme activities are due to the same enzyme, and also the fact that the THAL-NAD oxidoreductase activity curve coincides with that of acetaldehyde-NAD oxidoreductase (Fig. 1) seems to suggest that both enzyme activities are due to the same enzyme. If this is the case, TeHC may be a physiological substrate of the liver alcohol-NAD oxidoreductase and THAL may be a physiological substrate of the liver aldehyde-NAD oxidoreductase in rat liver.

Further confirmation of these hypotheses is now under investigation in our laboratory.

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