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HORSE LIVER 3α,7α,12α-TRIHYDROXY-5β-CHOLESTAN-26-AL DEHYDROGENASE AS A LIVER ALDEHYDE DEHYDROGENASE

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SUMMARY

3α,7α,12α-Trihydroxy-5β-cholestan-26-al dehydrogenase (3α,7α,12α-trihydroxy-5β-cholestan-26-al + NAD^+ + H_2O → 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid + NADH + H^+) in a soluble fraction of horse liver was studied.

1. Horse liver 3α,7α,12α-trihydroxy-5β-cholestan-26-al dehydrogenase consists of two components, the major one of which was purified about 70-fold by $(NH_4)_2SO_4$ fractionation, gel filtration, ion-exchange column chromatography and hydroxylapatite column chromatography.

2. 3α,7α,12α-trihydroxy-5β-cholestan-26-al dehydrogenase was always accompanied by liver aldehyde:NAD oxidoreductase (EC 1.2.1.3) activity, and the ratio of these two enzyme activities was not altered by purification.

3. Both 3α,7α,12α-trihydroxy-5β-cholestan-26-al dehydrogenase and horse liver aldehyde:NAD^+ oxidoreductase were reduced to a similar extent on inactivation of the purified enzyme by heating or by incubation with *p*-chloromercuribenzoate.

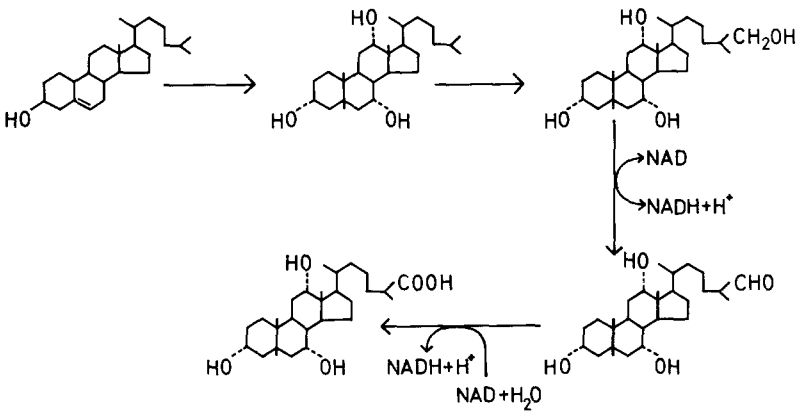
4. The K_m value for NAD^+ of 3α,7α,12α-trihydroxy-5β-cholestan-26-al dehydrogenase was found to be roughly equal to that of liver aldehyde:NAD^+ oxidoreductase.

5. The possibility was discussed that both 3α,7α,12α-trihydroxy-5β-cholestan-26-al dehydrogenase and liver aldehyde:NAD^+ oxidoreductase activities may be due to a single protein.

INTRODUCTION

In the conversion of cholesterol to cholic acid, an intermediate substance, 5β-cholestane-3α,7α,12α,26-tetrol (tetrahydroxycoprostane), is formed through the hydroxylation at C_{26} of 5β-cholestane-3α,7α,12α-triol, another intermediate formed by modifications of the nuclear part of cholesterol and which is the initial substrate for

Abbreviations: Tetrahydroxycoprostane, 5β-cholestane-3α,7α,12α,26-tetrol; trihydroxycoprostanal, 3α,7α,12α-trihydroxy-5β-cholestan-26-al; trihydroxycoprostanoic acid, 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid; PCMB, *p*-chloromercuribenzoate.



the successive side chain cleavage reactions[1–3]. We have found recently that tetrahydroxycoprostane is oxidized to 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (trihydroxycoprostanoic acid) by two successive dehydrogenases, tetrahydroxycoprostane dehydrogenase and 3α,7α,12α-trihydroxy-5β-cholestan-26-al (trihydroxycoprostanal) dehydrogenase, which are separable by gel filtration on Sephadex G-100 column[4,5]. Meanwhile it was found that these two steroid dehydrogenase activities are accompanied by those of liver alcohol dehydrogenase (alcohol:NAD^+ oxidoreductase, EC 1.1.1.1) and liver aldehyde dehydrogenase (liver aldehyde:NAD^+ oxidoreductase, EC 1.2.1.3)[5,6]. It was later shown on the basis of some criteria described by Dixon and Webb[7] that both tetrahydroxycoprostane dehydrogenase and liver alcohol dehydrogenase activities in rat liver are due to a single protein[8]. Similar experiments with rat liver trihydroxycoprostanal dehydrogenase were frustrated by the easy deterioration of rat liver enzyme during purification. Horse liver was, however, found to contain these dehydrogenases also and the aldehyde dehydrogenase was amenable to purification. Experiments reported in this paper suggest the identity of trihydroxycoprostanal dehydrogenase of horse liver with acetaldehyde dehydrogenase.

MATERIALS AND METHODS

Tetrahydroxycoprostane, trihydroxycoprostanal and trihydroxycoprostanoic acid were prepared according to the method described by Okuda and Danielsson[9]: the radioactive trihydroxycoprostanal was synthesized from radioactive cholic acid labeled by the method of Wilzbach[10]. NAD^+ and *p*-chloromercuribenzoate (PCMB) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Sephadex G-100 and DEAE-Sephadex A-50 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Kiesel Gel G and acetaldehyde were purchased from Merck Co. (Darmstadt, Germany). Hydroxylapatite was prepared according to the method described by Levin[11].

Trihydroxycoprostanal dehydrogenase activity was determined according to the following method: The substrate, ^3H-labeled trihydroxycoprostanal ($1.0 \cdot 10^7$ dpm/mg) was dissolved in methanol and the specified amount (50 μg in 20 μl) was pipetted into individual 25-ml test tubes. To this solution 1.0 ml of 0.1 M pyrophosphate buffer (pH 9.3) and 0.1 ml of 25 mM NAD^+ were added. The total volume was

brought to 3.0 ml by addition of appropriate amount of water. The mixture was then shaken in a bath, maintained at 37 °C, for a few minutes to equilibrate the temperature, and the reaction was started by addition of 10–100 μl of enzyme solution into this mixture. Incubations were conducted for 5 min at 37 °C with constant mechanical agitation. After 5 min 2 drops of 2 M HCl were added to the reaction mixture to stop the reaction and the solution was extracted with 5 ml of ethyl acetate. The aqueous layer was removed by pipetting and the extract was washed with water. The solvent was then evaporated by a stream of nitrogen gas. The residue was dissolved in 0.3 ml of ethyl acetate and the solution was applied on a thin-layer plate (not warmed) with Kiesel Gel as adsorbent. The plate was developed in phase system EA-2 [ethyl acetate–acetone (70:30, v/v)][12]. Tetrahydroxycholestane, trihydroxycholestanal and trihydroxycholestanoic acid were used as reference compounds. They were detected by spraying with 10% phosphomolybdic acid and the radioactivity on the plate was measured by scanning with a radiochromatogram scanner (Aloka Co., Tokyo, Japan, Model TLC-1). The percentage of an individual peak area was calculated and the amount of each product was obtained by multiplying the total amount of substrate incubated by this value and dividing by 100. One enzyme unit is defined as the number of μmoles of trihydroxycoprostanoic acid produced per min.

Liver aldehyde dehydrogenase activity was assayed by the method described by Racker[13]. Liver alcohol dehydrogenase was assayed as described before[8].

Purification of trihydroxycoprostanal dehydrogenase

The enzyme was prepared from a horse liver obtained within 1 h after slaughter, and all the subsequent procedures were performed in the cold room at 2–4 °C. 25 g of horse liver were homogenized in a Potter–Elvehjem homogenizer with 50 ml of 0.1 M phosphate buffer, pH 7.0. The homogenate was then centrifuged at 100 000 $\times$ g in a preparative ultracentrifuge (Hitachi Co., Tokyo, Japan, Model 55P-2) for 1 h. The supernatant was fractionated with $(NH_4)_2SO_4$. The precipitate obtained from the 0.2–0.6 $(NH_4)_2SO_4$ saturation was dissolved in the minimum amount of 0.035 M phosphate buffer (pH 7.8) and dialyzed against 0.007 M phosphate buffer (pH 7.8) for 20 h. The precipitate which had appeared after dialysis was removed by centrifugation at 9000 $\times$ g for 10 min and discarded. The supernatant (25 ml) was applied to a Sephadex G-100 column (3.0 cm $\times$ 140 cm) which had been equilibrated with 0.01 M phosphate buffer (pH 7.8), and eluted with about 2 l of 0.01 M phosphate buffer (pH 7.8) at a flow rate of 20 ml/h. Each 10.4 ml of the eluate was collected. Aliquots of each fraction were tested for both trihydroxycoprostanal dehydrogenase and liver aldehyde dehydrogenase activities. The fractions from 291 ml to 343 ml were combined and an aliquot (about one-third) was placed on a DEAE-Sephadex A-50 column (2.5 cm $\times$ 16 cm) which had been equilibrated with 0.01 M phosphate buffer (pH 7.8). The column was washed with the same buffer and eluted with 0.025 M and 0.075 M phosphate buffer (pH 7.8). The flow rate was 30 ml/h and each 7.3 ml of the eluate was collected. The effluents from 80 ml to 183 ml eluted by 0.075 M phosphate buffer were combined and put on a Sephadex G-25 column which had been equilibrated with 0.01 M phosphate buffer (pH 7.8) and eluted with the same buffer. The enzyme solution thus obtained was applied to a hydroxylapatite column (2 cm $\times$ 16 cm) which had been equilibrated with 0.01 M phosphate buffer (pH 7.8). The column was washed with the same buffer and eluted with 300 ml of phosphate

buffer (pH 7.8) applied as a linear gradient from 0.01 M to 0.2 M. The effluents from 210 ml to 260 ml were combined and applied to a Sephadex G-25 column which had been equilibrated with 0.01 M phosphate buffer (pH 7.8). The enzyme solution thus equilibrated with 0.01 M phosphate buffer was kept in the cold and used for the experiments within a few days.

Disc electrophoresis was performed according to the modified method of Davis[8,14].

RESULTS

Trihydroxycoprostanal dehydrogenase activities in 100 000 × g supernatant of horse liver homogenate and in the eluate of Sephadex G-100 column

Fig. 1a shows a typical thin-layer chromatogram of the products obtained by incubating ^{3}H-labeled trihydroxycoprostanal with horse liver 100 000 × *g* supernatant and NAD^+. As shown in the figure not only trihydroxycoprostanoic acid but also tetrahydroxycoprostane were produced by this incubation. This dismutation reaction is quite similar to those observed in 100 000 × *g* supernatant of rat liver[6] and human liver[15,16], and considered to be a coenzyme-linked dismutation as described by Racker[6,13]. It may be, therefore, highly difficult to determine a sole activity of liver

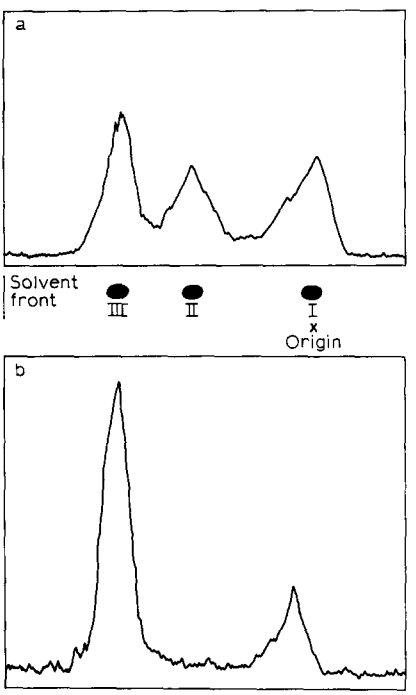


Fig. 1. Thin-layer chromatograms of the reaction products obtained by incubating trihydroxycoprostanal with NAD^+ and either 100 000 × *g* supernatant of horse liver (a) or Fraction 32 in (b). The reaction mixture contained 0.12 μmole trihydroxycoprostanal, 2.5 μmoles NAD^+, 100 μmoles phosphate buffer (pH 8.0) and 10 μl of enzyme solution. Incubations were conducted for 5 min at 37 °C. Solvent system: EA-2. I, trihydroxycoprostanoic acid; II, tetrahydroxycoprostane; III, trihydroxycoprostanal.

aldehyde dehydrogenase in the crude extract. On the other hand thin-layer chromatograms of the products obtained by incubating the steroid aldehyde with an aliquot of the eluate of Sephadex G-100 did not show any dismutation reaction as shown in Fig. 1b. These results are quite similar to those obtained with rat liver[6] and human liver[15,16].

Although trihydroxycoprostanoic acid did not move from the origin in this solvent system, it was confirmed by rechromatography with phase system S-7[17] that the material at the origin in these figures corresponds to trihydroxycholestanoic acid.

Trihydroxycoprostanal dehydrogenase and liver aldehyde dehydrogenase in the gel filtration eluate

As shown in Fig. 2, both enzyme activities in the gel filtration eluate of the 0.2–0.6 $(NH_4)_2SO_4$ fraction of horse liver extract were found in the same fraction and the profiles of the two activities coincided with each other. These results are again quite similar to those obtained with rat liver[6].

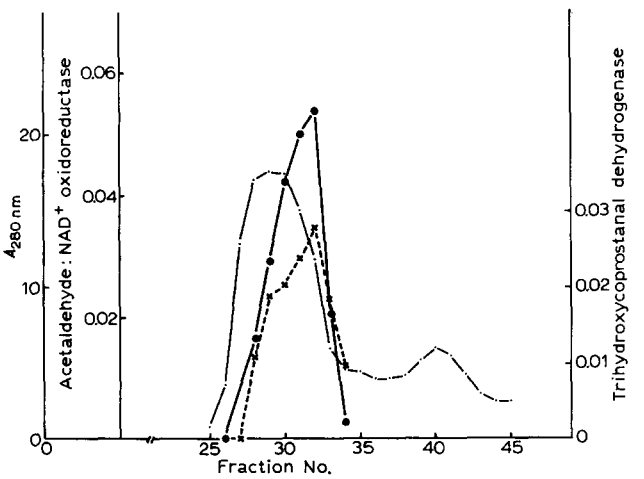


Fig. 2. Trihydroxycoprostanal dehydrogenase and liver aldehyde dehydrogenase activities in the eluate of gel filtration on Sephadex G-100 of $(NH_4)_2SO_4$ preparation (0.2–0.6 satn) of horse liver soluble fraction. Assays were performed as described in the text. —·—·, protein concentration ($A_{280\,nm}$); ×—×, trihydroxycoprostanal dehydrogenase (μmoles/5 min per 10 μl of fraction); ●—●, acetaldehyde dehydrogenase (absorbance increment/min per 100 μl of fraction).

Trihydroxycoprostanal dehydrogenase and liver aldehyde dehydrogenase in the eluate from DEAE-Sephadex

Fig. 3 shows the chromatogram obtained by the DEAE-Sephadex A-50 column. As shown in the figure, two peaks were noted in the chromatogram. One is the minor peak which appeared at lower ionic strength, the other is the major peak eluted at higher ionic strength. Although both enzyme activities were observed in these fractions, the following experiments were performed only with the enzyme from the major fraction, and no attempt was made to purify and characterize the enzyme in the minor peak at this time.

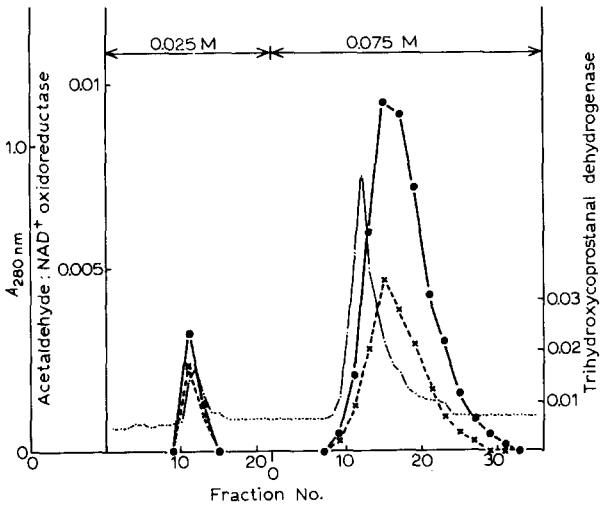


Fig. 3. Trihydroxycoprostanal dehydrogenase and liver aldehyde dehydrogenase activities in the eluate of DEAE-Sephadex column chromatography. Assays were performed as described in the text. —·—·, protein concentration ($A_{280\,nm}$); ×—×, trihydroxycoprostanal dehydrogenase (μmoles/5 min per 0.5 ml of fraction); ●—●, acetaldehyde dehydrogenase (absorbance increment/min per 0.5 ml of fraction).

Trihydroxycoprostanal dehydrogenase and liver aldehyde dehydrogenase in the eluate from the hydroxylapatite

Fig. 4 shows the chromatogram obtained from the hydroxylapatite column chromatography of the enzyme in the major fraction of Fig. 3. Both trihydroxycoprostanal dehydrogenase and acetaldehyde dehydrogenase activities were observed again in the same fraction, and the profiles of the two enzyme activities coincided well.

The extents of purification and ratios of trihydroxycoprostanal dehydrogenase activity to that of liver aldehyde dehydrogenase at each purification step

The specific activities and the yield of the two enzyme activities at each purification step are shown in Table I. Trihydroxycoprostanal dehydrogenase activities in the initial extract and $(NH_4)_2SO_4$ fraction were not assayed because the coenzyme linked dismutation described above would have made an accurate determination doubtful. However, it may well be assumed that the amount of enzyme recovered after gel filtration was roughly equal to that in the initial 100 000 × *g* supernatant. The average specific activity of both fractions in the crude extract was then calculated. If this value is compared to the specific activity of the hydroxylapatite eluate, a rough estimate of the overall purification is possible, and was found to be about 70-fold.

As shown in the table the ratio of trihydroxycoprostanal dehydrogenase activity and that of acetaldehyde dehydrogenase in the major fraction of DEAE-Sephadex eluate is equal to that in hydroxylapatite eluate, while specific activity of the latter increased. This implies that the ratio is not altered by purification procedures such as hydroxylapatite column chromatography.

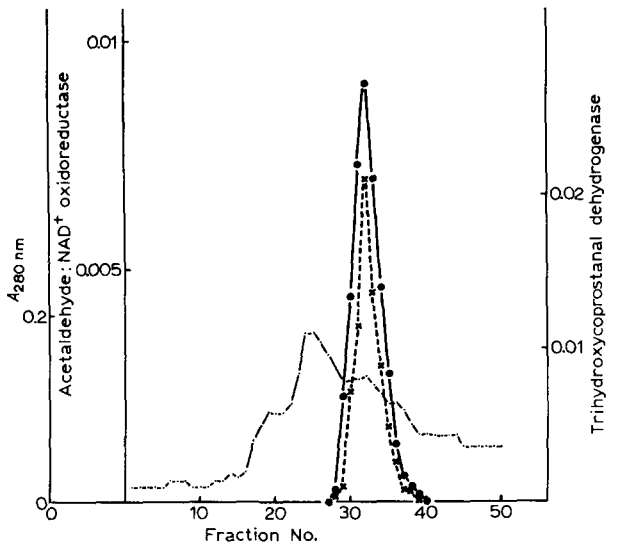


Fig. 4. Trihydroxycoprostanal dehydrogenase and liver aldehyde dehydrogenase activities in the eluate of hydroxylapatite column chromatography. Assays were performed as described in the text. —·—·, protein concentration ($A_{280\,nm}$); ×—×, trihydroxycoprostanal dehydrogenase (μmoles/5 min per 0.2 ml of fraction); ●—●, acetaldehyde dehydrogenase (absorbance increment/min per 0.5 ml of fraction).

TABLE I

SUMMARY OF ENZYME PURIFICATION

Enzyme activities were assayed as described in the text and the protein concentration was estimated by measuring the absorbance at 280 nm.

Step	*Volume (ml)*	*Total protein (mg)*	*Total units of aldehyde dehydrogenase*	*Specific activity (units/g protein)*	*Total units of trihydroxycoprostanal dehydrogenase*	*Specific activity (units/g protein)*	*Aldehyde dehydrogenase/trihydroxycoprostanal dehydrogenase*
Initial extract	45	2997	(3.09)	(1.03)	(4.83)	(1.61)	
$(NH_4)_2SO_4$ ppt	31	2579					
Sephadex G-100	58	800	3.09	3.86	4.83	6.04	
DEAE-Sephadex							
DEAE-Sephadex							
Fraction I	32	53	0.25	4.72	0.92	17.36	
Fraction II	265	106	2.35	22.17	2.81	26.51	0.84
Hydroxylapatite	84	26	1.95	75.00	2.20	84.61	0.89

Changes of trihydroxycoprostanal dehydrogenase and liver aldehyde dehydrogenase activities by inactivation treatment

Heat inactivation. The enzyme solutions were heated at 40, 50 and 60 °C for 10 min and then cooled rapidly in crushed ice. Aliquots of the enzyme solutions were tested for both trihydroxycoprostanal dehydrogenase and acetaldehyde dehydrogenase activities at the same time. As shown in Table II both enzyme activities were slightly affected by heating at 40 °C, whereas they were much reduced at 50 °C and

TABLE II

CHANGES OF TRIHYDROXYCOPROSTANAL DEHYDROGENASE AND ACETALDEHYDE DEHYDROGENASE ACTIVITIES BY THERMAL TREATMENT

Enzyme activities were assayed as described in the text after heating the enzyme solutions at various temperatures for 10 min.

No.	*Temperature (°C)*	*Trihydroxycoprostanal dehydrogenase*		*Acetaldehyde dehydrogenase*	
		units × 10^3	*%*	*units* × 10^3	*%*
1	2	5.26	100	3.57	100
2	40	4.63	88	3.14	88
3	50	2.86	54	1.88	53
4	60	0	0	0	0

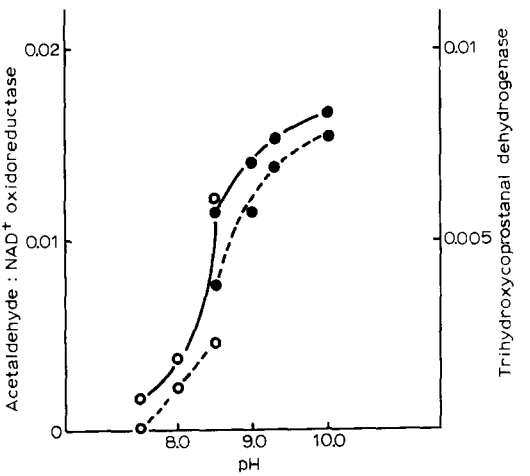


Fig. 5. Effect of pH on trihydroxycoprostanal dehydrogenase and liver aldehyde dehydrogenase activities. Assays were performed as described in the text except that Tris buffer (pH 7.5–8.5) or pyrophosphate buffer (pH 8.5–10.0) were used. ———, trihydroxycoprostanal dehydrogenase (μmoles/5 min per 0.3 ml of the enzyme); — — —, acetaldehyde dehydrogenase (absorbance increment/min per 0.4 ml of the enzyme).

TABLE III

CHANGES OF TRIHYDROXYCOPROSTANAL DEHYDROGENASE AND ACETALDEHYDE DEHYDROGENASE ACTIVITIES BY PCMB TREATMENT

Enzyme activities were assayed as described in the text after preincubating the enzyme solutions with various amounts of PCMB.

No.	*Concn of PCMB in preincubation mixture (μM)*	*Trihydroxycoprostanal dehydrogenase*		*Acetaldehyde dehydrogenase*	
		units × 10^3	*%*	*units* × 10^3	*%*
1	0	6.82	100	9.65	100
2	5	2.45	36	3.57	37
3	10	1.61	24	2.03	21
4	20	1.39	20	1.74	18
5	40	0.65	10	0.87	9

were completely lost at 60 °C. The losses of the two enzyme activities at each temperature were similar.

Inactivation by PCMB. The enzyme solutions were preincubated with various amounts of PCMB diluted to the appropriate extent, then trihydroxycoprostanal dehydrogenase and acetaldehyde dehydrogenase were assayed at the same time. Trihydroxycoprostanal was incubated at room temperature for 10 min in this case to avoid any further inactivation during the period of activity assay. Both enzyme activities were reduced to a little over one-third of the original activities by treatment with 5 μM PCMB and almost completely abolished by treatment with 40 μM PCMB (Table III). The extent of the reduction of both enzyme activities was roughly equal at each PCMB concentration.

Effect of pH on both trihydroxycoprostanal dehydrogenase and liver aldehyde dehydrogenase

To compare the effect of pH on both trihydroxycoprostanal dehydrogenase and acetaldehyde dehydrogenase activities, each substrate was incubated in media of different pH. As shown in Fig. 5, both enzyme activities increased continuously from pH 7.5 to pH 10.0, and the profiles of pH–activity curves of the two enzyme activities are similar.

TABLE IV

MICHAELIS CONSTANTS OF HORSE LIVER TRIHYDROXYCOPROSTANAL DEHYDROGENASE FOR VARIOUS SUBSTRATES

Michaelis constants (K_m) were estimated from Lineweaver–Burk plots.

Assay method	K_m (μM)		
	NAD^+	*Trihydroxy-coprostanal*	*Acetaldehyde*
Trihydroxycoprostanaldehydrogenase	132	41.6	—
Acetaldehyde dehydrogenase	102	—	2.3

K_m values for NAD^+, trihydroxycoprostanal and acetaldehyde

Table IV shows the K_m values for NAD^+, trihydroxycoprostanal and acetaldehyde. As shown in the table K_m for NAD^+ of trihydroxycoprostanal dehydrogenase is equal in order to that of acetaldehyde dehydrogenase. A slight difference may be due to the fact that the concentration of trihydroxycoprostanal could not be raised sufficiently to saturate the enzyme. The enzyme activity was not observed when $NADP^+$ was substituted for NAD^+. On the other hand K_m for trihydroxycoprostanal is much higher than that for acetaldehyde.

An attempt to compare K_i (the inhibitor constant) values for chloral hydrate, a known competitive inhibitor of the enzyme, was unsuccessful because non-enzymatic alkaline interaction between trihydroxycoprostanal and the inhibitor was inevitable, and an unidentified new product, which is less polar than any of three reference compounds, trihydroxycoprostanoic acid, trihydroxycoprostanal and tetrahydroxycoprostane, appeared in the incubation of trihydroxycoprostanal regardless of whether the enzyme was present or not. This was also the case with another known inhibitor, trichloroacetaldehyde.

Reversibility

Incubation of ^{3}H-labeled trihydroxycoprostanoic acid with the enzyme and NADH at different pH values did not result in any detectable formation of trihydroxycoprostanal.

Disc electrophoresis of trihydroxycoprostanal dehydrogenase and liver aldehyde dehydrogenase

The eluate from the hydroxylapatite column was subjected to disc electrophoresis, and the enzyme activities were located by either staining for acetaldehyde dehydrogenase activity or by assaying trihydroxycoprostanal dehydrogenase activity after eluting the sliced gel with a minimum amount of 0.01 M phosphate buffer (pH 7.0). The eluate from the hydroxylapatite column gave a single band staining for acetaldehyde dehydrogenase activity. Furthermore, the same band contained the trihydroxycoprostanal dehydrogenase activity. Neither of the two enzyme activities was found anywhere else.

DISCUSSION

Trihydroxycoprostanal dehydrogenase of horse liver consists of two fractions, one of which is the minor fraction eluted in lower ionic strength on DEAE-Sephadex column chromatography, the other is the major fraction eluted in higher ionic strength. Both fractions were accompanied by acetaldehyde dehydrogenase activity. On further purification of the major fraction by hydroxylapatite column chromatography the acetaldehyde dehydrogenase activity was not lost. Furthermore, both enzyme activities were reduced to a similar extent by heat inactivation or PCMB treatment. K_m values for NAD^+ of the two enzymes were in the same order of magnitude whereas the K_m value for trihydroxycoprostanal differed from that of acetaldehyde. The enzyme solution purified by hydroxylapatite column chromatography gave a single band on staining for acetaldehyde dehydrogenase when subjected to disc electrophoresis, where trihydroxycoprostanal dehydrogenase activity was also observed. All these results suggest that trihydroxycoprostanal dehydrogenase and acetaldehyde dehydrogenase activities are due to a single protein, and that one of the natural substrates of liver aldehyde dehydrogenase is trihydroxycoprostanal.

The present results, together with those previously reported that liver tetrahydroxycoprostane dehydrogenase and liver alcohol dehydrogenase are the same enzyme[8] suggest that both liver alcohol dehydrogenase and liver aldehyde dehydrogenase are functioning in the side chain oxidizing reactions in cholesterol metabolism in the liver as indicated in the diagram.

It was found in this laboratory recently that human liver has properties similar to those of rat and horse liver in respect to these two enzymes. So it may be suggested that cholesterol metabolism indicated in the diagram is also functioning in human liver.

During the preparation of this paper the purification of horse liver aldehyde dehydrogenase by a different method was reported by Feldman and Weiner[18], who also noticed the two types of heterogeneity.

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