Human Liver–Alcohol Dehydrogenase. Kinetic and Physicochemical Properties*

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Received July 27, 1964

A procedure for the purification of an alcohol dehydrogenase from human liver is detailed. The DPN(H)-dependent human liver–alcohol dehydrogenase exhibits the functional characteristics of a metalloenzyme and contains about 2 g-atoms of zinc per provisional molecular weight 87,000. The human enzyme exhibits distinctive substrate specificity. The human enzyme oxidizes methanol and ethylene glycol, thus providing a rational basis for the heretofore empirical use of ethanol in methanol or ethylene glycol poisoning. The values of the physicochemical and kinetic parameters are similar to those of the enzyme isolated from horse liver.

The alcohol dehydrogenase of human liver, like that from yeast and horse liver, is a DPN-dependent zinc metalloenzyme (Vallee and Hoch, 1957). The present study demonstrates that the properties of human liver–alcohol dehydrogenase resemble those of the horse enzyme as regards metal content, inhibition by metal-binding agents, stoichiometry of coenzyme binding, spectral characteristics of the LADH–DPNH complex, and in physicochemical properties.[1] They differ, however, in regard to specificity.

Materials and Methods

Reagents.—The preparation of metal-free distilled water, metal-free buffers, and glassware was carried out as previously described (Thiers, 1957). The reagents for the isolation procedure were reagent grade and used without further purification. DPN^+ was obtained from Pabst Laboratory, DPNH from Sigma Chemical Co. Reagent-grade 95% ethanol was used without further purification and acetaldehyde (Eastman Organic Chemicals) was freshly distilled before use. The purity of methanol (Spectranalyzed, Fisher Scientific Co.) was documented by gas-chromatographic analysis. No alcohols other than methanol were detected in the sample. All other alcohols employed to test the specificity of the enzyme were reagent grade and were used without further purification. Compounds used for inhibition studies were reagent grade.

Enzymatic Activity.—The catalytic activity of human liver–alcohol dehydrogenase was measured spectrophotometrically at 23°, the rate of DPNH formation being followed at 340 mμ in a Beckman DU spectrophotometer. The enzymatic reaction was initiated by addition of 0.1 ml of enzyme solution to 3 ml of assay mixture, consisting of 1.6×10^{-2} M sodium pyrophosphate buffer, *p*H 8.8, 1.6×10^{-2} M DPN, and 1.6×10^{-2} M ethanol. The concentration of the enzyme solution added was adjusted to result in a change of absorbance, ΔA, of 0.1/min at 340 mμ. For all steps in the enzyme preparation a "blank" reaction containing no ethanol was run as a control. Alcohol-dehydrogenase activity was calculated as the difference in the ΔA_{340}/min of the two reactions, with and without ethanol. The specific activity is defined as ΔA_{340}/min per A_{280}, where A_{280} is the absorbance of the solution at 280 mμ. The "blank" reaction becomes negligible after dialysis to remove ammonium sulfate and was omitted in measurements subsequent to this step.

Metal Analyses.—Metal content was determined by emission spectrography (Vallee, 1955). Zinc determinations were also carried out by atomic-absorption spectrophotometry (Fuwa and Vallee, 1963). Before determination of metal content all samples were dialyzed against metal-free distilled water or buffer.

Protein Concentration.—Protein concentration was determined by triple trichloroacetic acid precipitation (Hoch and Vallee, 1953). The absorbance at 280 mμ was determined for each enzyme preparation and served for subsequent spectrophotometric measurements of the protein concentration. For the most highly purified enzyme, the absorbance of a 1 mg/ml solution was 0.46/cm in 0.03 M phosphate, 0.07 M NaCl, *p*H 7.0.

Inhibition by Chelating Agents.—Solutions of ethylenediaminetetraacetic acid, 8-hydroxyquinoline, 1,10-phenanthroline, sodium cyanide, sodium sulfide, α,α-dipyridyl, and *l*-thyroxin were prepared, the *p*H was adjusted to 8.8, and an aliquot was added directly to the reaction mixture. The enzyme was then added to initiate the reaction.

Continuous spectra of DPNH solutions in the presence of enzyme were determined in a Cary Model 15 recording spectrophotometer.

Sedimentation in a Spinco Model E ultracentrifuge was carried out between 20 and 22° at 59,780 rpm. The temperature was measured by means of the RTIC unit and the data were corrected accordingly (Oncley, 1941).

Molecular weights were determined using the method of Klainer and Kegeles (1955). A partial specific volume of 0.75 ml/g was assumed.

Electrophoreses in a Spinco Model H electrophoresis and diffusion apparatus were carried out at 1°, at a protein concentration of 10 mg/ml in fields of 1–3 v/cm. Mobilities were calculated from movements of the maximum of the schlieren peak in the descending limb.

Conductivities were determined in an ice-water bath using a Radiometer conductivity meter. Mobilities were therefore referred to 0°.

Purification Procedure.—A schematic diagram of the fractionation procedure is shown in Figure 1. Livers were obtained at autopsy from patients of both sexes, whose ages ranged from 19 to 82 years, and who died of arteriosclerotic or other forms of heart disease. In all

* This work was supported by the Howard Hughes Medical Institute, by grants-in-aid (HE-07297) from the National Institutes of Health of the Department of Health, Education and Welfare, and by the Nutrition Foundation. Preliminary accounts have been presented at the 143rd Meeting of the American Chemical Society (Von Wartburg and Vallee, 1963; Bethune *et al.*, 1963).

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[1] Abbreviation used in this work: LADH, liver-alcohol dehydrogenase.

TABLE I
PURIFICATION OF ALCOHOL DEHYDROGENASE FROM HUMAN LIVER

Step of Purification	Volume (ml) A	Specific Activity ΔA_{340}/min/ A_{280} B	Specific Activity ΔA_{340}/min/ g Protein C	LADH Activity (ΔA_{340}/min) D	Yield (% of initial) E
(1) Filtrate	1620	0.014	4.2	720	100
(2) Supernatant III	360	0.041	75.6	670	93
(3) Eluate I	67	0.129	242	704	98
(4) Eluate II	105	1.21	856	637	88

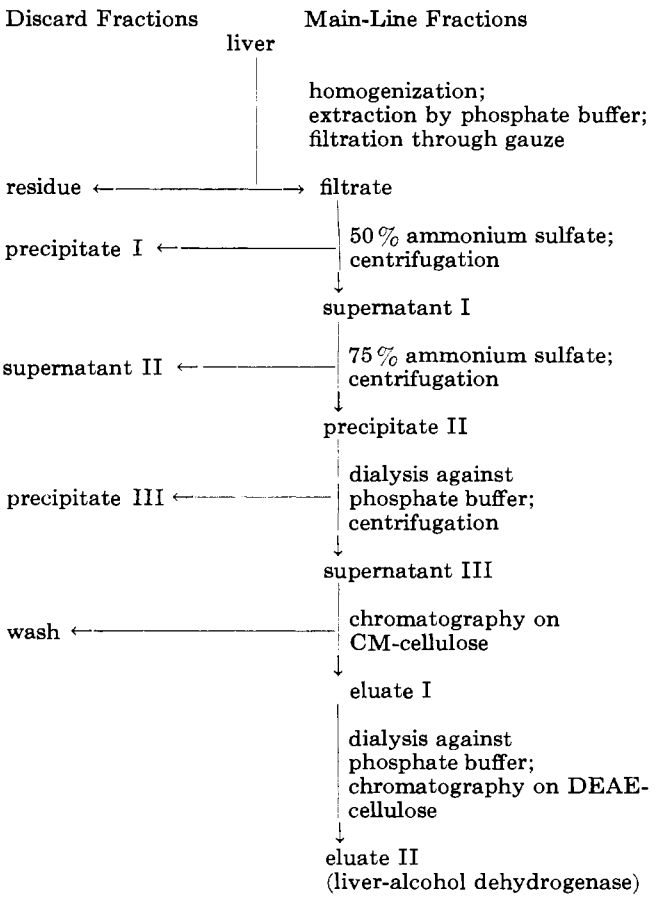

FIG. 1.—Purification of human liver–alcohol dehydrogenase (flow sheet).

instances, the case history and the pathological criteria indicated the liver to be free of disease. The livers were frozen as soon as possible.

On removal from storage 1 kg of liver is thawed overnight at 4°, then rinsed to remove blood and passed through a meat grinder. The homogenate is extracted at 4° with twice its own volume of 0.05 M sodium phosphate buffer, *p*H 7.0, with continuous fast stirring followed by filtration through gauze. All subsequent steps are carried out at 4°. To the filtered extract, about 2200 ml, an equal volume of saturated ammonium sulfate solution is added rapidly. The *p*H of the ammonium sulfate solution is adjusted with concentrated ammonia to 7.0, as measured after dilution of an aliquot of the solution to 0.2 M. The precipitate (precipitate I) is removed by centrifugation after 1 hour (IEC, 850 head, 3800 rpm, 45 minutes) and an equal volume of saturated ammonium sulfate solution is added to the clear supernatant solution (supernatant I). The red precipitate (precipitate II) is collected by centrifugation for 1 hour and transferred to dialysis tubing using a minimum volume of 0.05 M sodium phosphate buffer, *p*H 7.0. Dialysis is carried out first against two 4-liter changes of the same buffer and then against three 4-liter changes of 0.005 M sodium phosphate buffer, *p*H 7.0. The dark-red solution, about 350 ml, is centrifuged and the precipitate (precipitate III) is discarded.

The solution (supernatant III) is run into a CM-cellulose column, 2.4 cm in diameter and 60 cm high, prepared according to Peterson and Sober (1956), after prior equilibration of the column with 0.005 M sodium phosphate buffer, *p*H 7.0. Equilibration buffer is then run through until the absorbance of the effluent at 280 mμ is less than 0.2. Under these conditions little enzymatic activity is lost and contaminating proteins emerge in the red-brown effluent. The enzyme, together with residual hemoglobin, is eluted in a single step with 0.05 M sodium phosphate buffer, *p*H 7.0, to yield eluate I. The enzyme activity runs at the forward edge of the dark-red band of hemoglobin.

The enzyme solution is then dialyzed against two 1-liter changes of 0.002 M sodium phosphate buffer, *p*H 7.8, followed by chromatography on a DEAE-cellulose (Eastman) column prepared in the same manner as the CM-cellulose column and equilibrated against the above buffer. After running in the solution, the column is washed with the equilibration buffer. Hemoglobin is bound while the enzyme is eluted as a colorless solution, appearing in a volume of about 60 ml and constituting eluate II. Further purification of the enzyme was achieved by rechromatography on CM-cellulose.

RESULTS

The results of a typical enzyme purification are summarized in Table I. The specific activity (columns B, C) rises approximately 200-fold during purification. The final yield of this preparation (column E, line 4) was 88% of the total enzymatic activity of the initial extract (column B, line 1). The yield of a series of different preparations ranged from 41 to 88%.

The total activity per gram of liver in the extract, in a series of preparations, ranged from 0.6 to 1.5, averaging 1.1 activity units (ΔA_{340}) per minute.

Metal Analyses.—Table II presents spectrographic

TABLE II
METAL CONTENT OF DIFFERENT PREPARATIONS OF HUMAN LIVER–ALCOHOL DEHYDROGENASE BY EMISSION SPECTROGRAPHY AND ATOMIC ABSORPTION[a]

Preparation	Zinc (spectrographic)	Zinc (atomic absorption)	Iron	Aluminum
I	2.2	[b]	0.22	0.18
II	1.9	[b]	0.39	0.45
III	2.1	2.1	[c]	[c]
IV	2.5	2.5	[c]	[c]

[a] Metal contents are in g-atoms of metal per 87,000 g of protein. [b] Not done. [c] Not detected; also Ba, Ca, Cd, Co, Cr, Li, Mg, Mn, Mo, Ni, Pb, Sn, Sr were not detected in any sample.

TABLE III
ACTIVITY AND METAL CONTENT OF FRACTIONS ATTENDING PURIFICATION OF HUMAN LIVER–ALCOHOL DEHYDROGENASE[a]

Step of Purification	Specific Activity A	Zinc/Protein B	Zinc/Protein C	Activity/ Zinc D	Other Metals Fe E	Ca F	Mg G	Al H	Ba I	Cr J	Mn K	Pb L	Sr M	ΣMe N
(1) Filtrate	4.2	820	1.0	0.5	2.7	1.1	3.3	0.044	0.009	0.031	0.023	0.009	0.013	7.23
(2) Supernatant I	75.6	810	1.0	9.3	2.0	0.20	<0.13	0.044	0.023	<0.006	0.014	0.011	[b]	2.43
(3) Eluate I	242	880	1.1	27.6	3.0	0.20	0.19	0.030	0.001	0.012	0.010	[b]	[b]	3.44
(4) Eluate II	834	1900	2.3	45.0	0.14	0.40	0.33	[b]	[b]	0.010	0.014	[b]	0.007	0.90
(5) Eluate II, dialyzed	856	1700	2.1	50.2	0.14	0.10	<0.24	[b]	[b]	0.013	0.006	[b]	0.016	0.52
(6) Rechromatographed on CM-cellulose and dialyzed	975	1700	2.1	57.0	[b]	0.009	[b]	[b]	[b]	[b]	[b]	[b]	[b]	0.01

[a] The metal contents are in g-atoms of metal per 87,000 g of protein; column B in μg/g. Activities are in ΔA_{340}/min per g of protein (column A) or ΔA_{340}/min per 100 μg of zinc (column D). [b] Not detected. Also Cd, Co, Li, Mo, Ni, Sn were not detected in any sample.

TABLE IV
EFFECT OF METAL-BINDING AGENTS ON ALCOHOL-DEHYDROGENASE ACTIVITY[a]

Agent	Concentration (M)	V_i/V_c
NaN_3	1.66×10^{-1}	0.63
	2×10^{-1}	0.59
	2.33×10^{-1}	0.54
	3.33×10^{-1}	0.40
Na_2S	1.66×10^{-3}	0.76
	3.33×10^{-3}	0.59
	8.33×10^{-3}	0.40
	1.66×10^{-2}	0.25
EDTA	3.33×10^{-2}	0.85
	5×10^{-2}	0.70
	6.66×10^{-2}	0.61
	8.33×10^{-2}	0.47
α,α'-Dipyridyl	3.3×10^{-3}	0.84
	6.66×10^{-3}	0.63
l-Thyroxin	1.66×10^{-4}	0.63
	2.5×10^{-4}	0.57
	2.9×10^{-4}[b]	0.53

[a] Activity is measured by adding 0.1 ml of the enzyme solution to a 3-ml reaction cuvet containing 1.66×10^{-2} M sodium pyrophosphate, *p*H 8.8, 1.66×10^{-3} M DPN, and 1.66×10^{-2} M ethanol and the appropriate concentration of the agent; the change in absorbance at 340 mμ/min is measured. [b] Saturated solution.

analyses of four different preparations of human liver–alcohol dehydrogenase. Analyses were performed on materials after chromatography on DEAE-cellulose (I and II) and after rechromatography on CM-cellulose (III and IV). The molar ratio of the major metallic constituent, zinc, to protein varies from 1.9 to 2.5, averaging 2.2, based on a molecular weight of 87,000 (*vide infra*). Of other metals, only aluminum and iron were present in detectable amounts, but the stoichiometric amount of these metals is insignificant and varies widely from one preparation to another.

The relationship of zinc to enzymatic activity is shown in Table III. The metal content and enzymatic activity of consecutive fractions obtained during a preparation (preparation III of Table II) is shown. Zinc, iron, calcium, and magnesium are the only metals which are present in stoichiometrically significant quantities in the initial extract (line 1, columns C, E, F, G). Specific activity rises progressively from 4.2 units/g of protein in the initial extract to 975 units/g in the final product (column A). The zinc content also rises from 820 to 1700 μg/g of protein (column B) or from 1.0 to 2.1 g-atoms of zinc per mole of protein (column C), concomitant with an increase of the ratio of enzymatic activity/zinc (column D) from 0.5 to 57.0 units/100 μg of zinc.

In contrast, the *individual* concentration of each of the other elements (columns E–M), as well as their sum, ΣMe, (column N), *decreases* with purification. In only a few instances does the concentration of any metal increase between purification steps. Iron (column E) increases after CM-cellulose chromatography, but, as might be expected, this element was removed together with hemoglobin upon chromatography on DEAE-cellulose. Both calcium (column F) and magnesium (column G) are increased after chromatography on CM-cellulose, suggesting the presence of these elements in the buffer. Their concentrations are sharply reduced in the final purification step (line 6).

Inhibition by Metal-binding Agents—While the presence of a metal atom as an intrinsic part of the molecule is demonstrated by the analytical data, its involvement in activity must be demonstrated by inhibition studies with chelating agents. Indeed, when

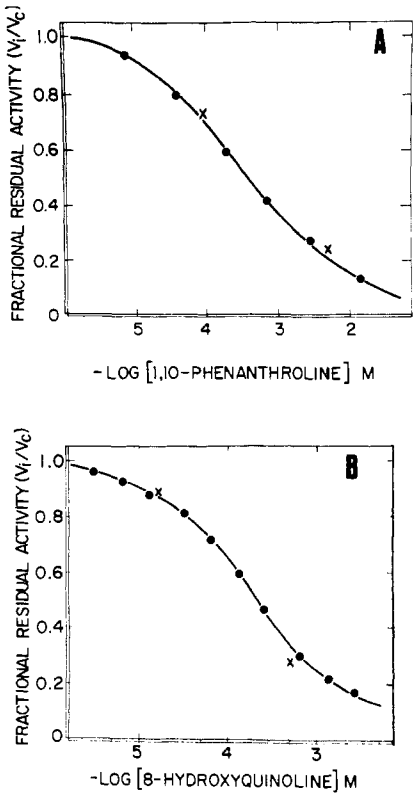

FIG. 2.—The instantaneous, reversible inhibition of human liver-alcohol dehydrogenase with 1,10-phenanthroline (A) and 8-hydroxyquinoline (B). Fractional residual activity (V_i/V_c) of the enzyme versus the negative logarithm of the inhibitor concentration. (V_c is the uninhibited control activity, V_i is the inhibited activity.) The assay solution contained: DPN$^+$, 1.66×10^{-3} M; ethanol, 1.66×10^{-2} M, 0.1 M sodium pyrophosphate, *p*H 8.8; 23°. The crosses (×) represent partial activity when a reaction mixture containing 1,10-phenanthroline (5×10^{-3} M) or 8-hydroxyquinoline (5×10^{-4} M) is diluted after the activity measurements (V_i/V_c = 0.23 or 0.27, respectively) to produce a final 1,10-phenanthroline or 8-hydroxyquinoline concentration of 8.33×10^{-5} M or 1.66×10^{-5} M, respectively. Only enzyme and inhibitor are diluted.

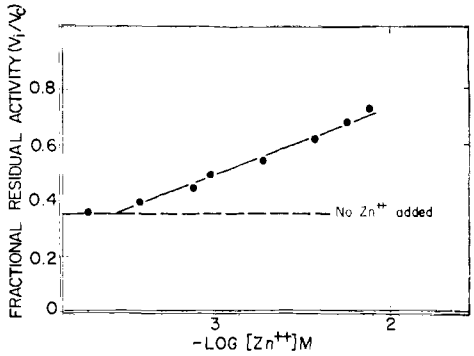

FIG. 3.—The effect of varying zinc concentration on the inhibition of alcohol-dehydrogenase activity by 1,10-phenanthroline. Fractional residual activity (V_i/V_c) as a function of the negative logarithm of the zinc-ion concentration at a constant concentration of 1×10^{-3} M 1,10-phenanthroline in the reaction cuvet. DPN$^+$, 1.6×10^{-3} M, ethanol, 1.6×10^{-2} M, 3.3×10^{-2} M sodium pyrophosphate, *p*H 8.8; 23°.

metal-binding agents are added to the assay mixture *before* the addition of the enzyme, an instantaneous inhibition of the enzymatic activity of human liver-alcohol dehydrogenase is observed. A list of these agents, together with the degree of inhibition (V_i/V_c)

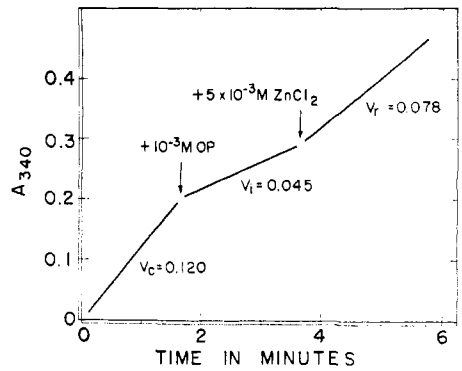

FIG. 4.—Effect of zinc on the inhibition of alcohol-dehydrogenase activity. Absorbance at 340 mμ as a function of time. The reaction is initiated by addition of enzyme to a solution containing DPN$^+$, 1.6×10^{-3} M; ethanol, 1.6×10^{-3} M; 1.6×10^{-2} M sodium pyrophosphate, *p*H 8.8; 23°. Reagents are added as indicated in the figure. Absorbances are corrected for dilution and loss of activity of the uninhibited enzyme. V_c, control activity; V_i, inhibited activity; V_r, restored activity.

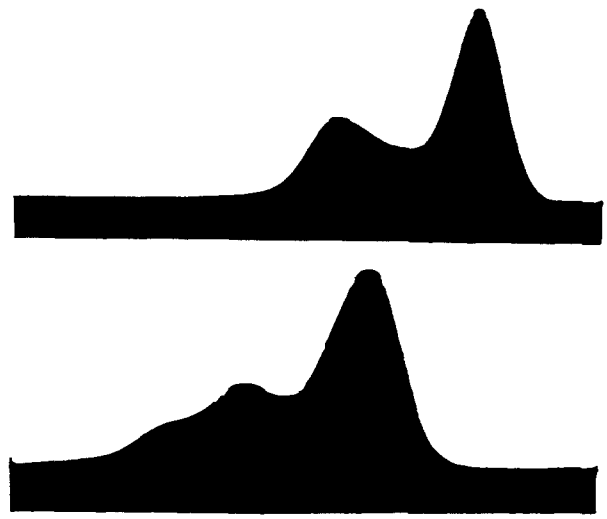

FIG. 5.—Electrophoretic patterns of two human liver-alcohol dehydrogenase preparations after 16 hours at 2 v/cm. Upper pattern: 0.02 M sodium barbital, 0.08 M NaCl, *p*H 8.0. Lower pattern: 0.03 M sodium phosphate, 0.07 M NaCl, *p*H 7.4. The anode is on the right. The direction of motion of the main boundary is from left to right. The exposures were taken just before sampling, and the boundaries have been compensated to a favorable position.

at different concentrations of the chelating agent, is given in Table IV. The relative activity as a function of the logarithm of the concentration of the inhibitor is shown in Figure 2 for two other chelating agents, 8-hydroxyquinoline and 1,10-phenanthroline.

The instantaneous inhibition of human liver-alcohol dehydrogenase by both 1,10-phenanthroline (Fig. 2A) and 8-hydroxyquinoline (Fig. 2B) is completely reversible on dilution. In an assay mixture containing 5×10^{-3} M 1,10-phenanthroline the enzyme exhibits 23% of the control activity (V_i/V_c = 0.23). When this mixture is diluted with a solution containing all components of this system, except the inhibitor, reversal of the inhibition is apparent as evident from an increase of the ratio V_i/V_c. The enzyme shows 73% of its full activity when the concentration of 1,10-phenanthroline has been diluted to 8.33×10^{-5} M. This percentage is exactly the same as that obtained when 8.33×10^{-5} M 1,10-phenanthroline is added directly to the assay mixture. The same is true of the inhibition

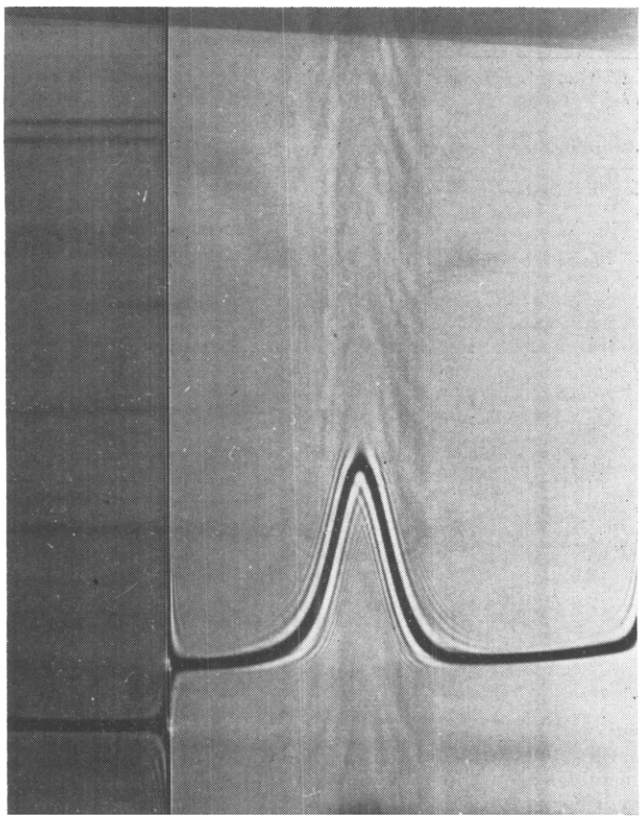

FIG. 6.—Sedimentation pattern of human liver–alcohol dehydrogenase after 60 minutes at 59,780 rpm in 0.03 M sodium phosphate, 0.07 M NaCl, *p*H 7.0; 20–22°. Protein concentration, 19 mg/ml. Direction of sedimentation is from left to right.

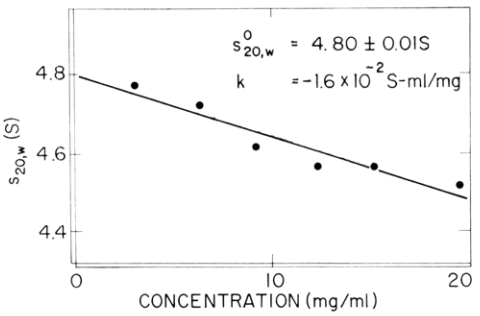

FIG. 7.—Concentration dependence of the sedimentation coefficient ($s^\circ_{20,w}$) of human liver–alcohol dehydrogenase. Conditions: 0.03 M sodium phosphate, 0.07 M NaCl, *p*H 7.0; 20–22°. Each point was calculated by least squares, as was the solid line.

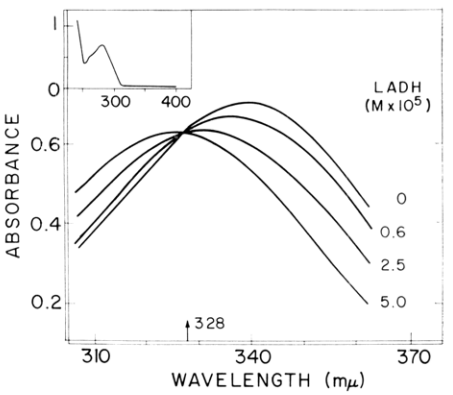

FIG. 8.—Spectra of DPNH in the presence of varying concentrations of human liver–alcohol dehydrogenase in 2×10^{-3} M sodium phosphate, *p*H 7.8. The enzyme concentration is as indicated, that of DPNH is 1.13×10^{-4} M. The insert is the spectrum of the enzyme alone.

by 8-hydroxyquinoline. Thus the reaction between human liver–alcohol dehydrogenase and either 1,10-phenanthroline or 8-hydroxyquinoline, causing instantaneous inhibition, appears to reach a rapid and freely reversible equilibrium, as is the case in the horse enzyme (Vallee *et al.*, 1959).

If this equilibrium is established rapidly, then competition for the chelating agent should be demonstrable between added extrinsic zinc ions and the intrinsic zinc of the enzyme. Indeed, Zn^{2+} ions *decrease* the inhibition of human liver–alcohol dehydrogenase activity caused by 10^{-3} M 1,10-phenanthroline (Fig. 3). This concentration of 1,10-phenanthroline in the reaction mixture results in 35% of the control activity; when zinc ions at a concentration of 1×10^{-2} M are added, 74% of the initial activity is found. When the concentration of zinc is increased further, precipitation occurs. Increase of activity is directly proportional to the logarithm of the concentration of Zn^{2+}, up to 10^{-2} M zinc chloride.

The inhibition with 1,10-phenanthroline, once established, can also be *reversed* by addition of Zn^{2+}. Thus, as shown in Figure 4, the addition of 1,10-phenanthroline to a reaction mixture immediately inhibits the rate while the subsequent addition of Zn^{2+} ions restores it.

Electrophoresis.—Patterns of two preparations after the DEAE-cellulose chromatographic step exhibit two boundaries (Fig. 5). At *p*H 7.4, the mobility of the small peak was $+0.55 \times 10^{-5}$ cm^2 v^{-1} sec^{-1}, that of the large peak was -0.80×10^{-5} cm^2 v^{-1} sec^{-1}. Sampling of the electrophoresis cell at the end of the runs showed the large peak to be the active fraction, the small peak the inactive. The initial specific activity of the sample represented by the upper pattern was 1.29, that for the lower 1.87 units. The large peak had an activity of 2.2 units after electrophoresis.

Sedimentation.—At all protein concentrations studied one boundary was observed during sedimentation (Fig. 6).

The values of the sedimentation coefficient as a function of concentration are shown in Figure 7. Fitting the data to the equation: $s_{20,w} = s^0_{20,w}(1 - kC)$ where $s_{20,w}$ is the sedimentation coefficient found at concentration C, a value of 4.80 ± 0.01 S for $s^0_{20,w}$ and a slope, k, of 3.4×10^{-3} S-ml mg^{-1} were found.

Molecular Weight.—Determination of the molecular weight by a modification of the Archibald procedure (Klainer and Kegeles, 1955) gave a molecular weight of 87,000. Although sedimentation studies of another preparation showed only one boundary (Fig. 6), the time course of the molecular-weight determination showed some heavy material to be present in this preparation. The molecular weight found, therefore, requires confirmation.

Coenzyme Binding.—On binding of DPNH to human liver–alcohol dehydrogenase the absorption maximum of free DPNH at 340 mμ undergoes hypsochromic and hypochromic shifts to 325 mμ (Fig. 8) similar to the horse enzyme (Theorell and Bonnichsen, 1951). The difference in the coefficient of absorptivity between free and bound DPNH is maximal at 355 mμ and an isosbestic point occurs at 328 mμ. DPNH was not present in the final enzyme preparation, as judged by the absence of absorption characteristic of DPNH (Fig. 8, insert).

The stoichiometry of DPNH binding by alcohol dehydrogenase was determined by spectral titration

TABLE V
MICHAELIS CONSTANTS OF HUMAN LIVER-ALCOHOL DEHYDROGENASE FOR COENZYMES AND SUBSTRATES[a]

	Buffer	pH	Concentration of Coenzyme or Substrate (M)	K_m (M)
DPN^+	Sodium pyrophosphate, 1.66×10^{-2} M	8.8	1.66×10^{-2}	1.1×10^{-4}
Ethanol	Same	8.8	1.66×10^{-3}	1.2×10^{-3}
DPNH	Sodium phosphate, 3.3×10^{-2} M	7.5	5.0×10^{-2}	2.2×10^{-5}
Acetaldehyde	Same	7.0	3.3×10^{-4}	4.3×10^{-4}

[a] Measured at the indicated concentration of coenzymes or substrates at 23° in the buffer and at the pH indicated.

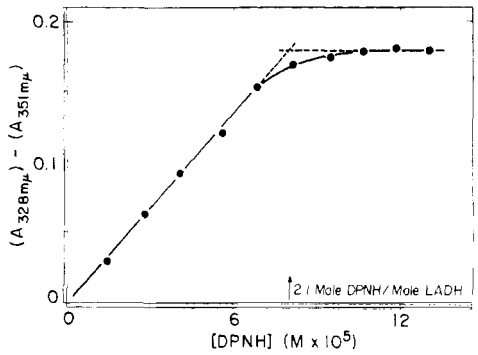

FIG. 9.—Spectral titration of human liver-alcohol dehydrogenase with DPNH in 5×10^{-2} M sodium phosphate, pH 7.0. Increasing concentrations of DPNH are added to 3.8×10^{-5} M enzyme.

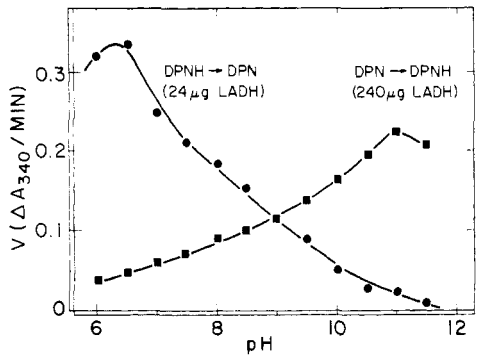

FIG. 10.—pH dependence of ethanol oxidation (■) and acetaldehyde reduction (●) for human liver-alcohol dehydrogenase. Conditions: pH 6.0–8.0, 3.3×10^{-2} M sodium phosphate; pH 8.0–9.0, 3.3×10^{-2} M Tris-HCl; pH 9.0–11.5, 3.3×10^{-2} M glycine-NaOH. DPNH, 3.3×10^{-3} M; acetaldehyde, 8.3×10^{-2} M; or DPN^+, 1.6×10^{-3} M; ethanol, 1.6×10^{-2} M; 23°.

in which increasing amounts of DPNH were added to a solution of enzyme (Fig. 9). The concentration of bound DPNH was measured by the difference of the optical absorbances at 328 and 351 mμ. The amount of bound DPNH correlates closely to the specific activity of a given preparation as would be expected if impurities do not bind the coenzyme. In accord with expectations, the inactive protein separated by free-boundary electrophoresis did not bind DPNH. For the most highly purified preparations a mole ratio of 2 moles of DPNH bound per mole of enzyme was obtained.

Kinetic Constants.—The Michaelis constants of human liver-alcohol dehydrogenase with DPN^+ and ethanol, DPNH and acetaldehyde, calculated from Lineweaver-Burk plots by the method of least mean squares, are given in Table V. The specific activity of the enzyme preparations used for these determinations ranged from 0.9 to 1.5. Repeated determinations with enzyme preparations from different livers were in close agreement.

Initial velocities for the oxidation of ethanol and the reduction of acetaldehyde were measured from pH 6 to 11.5 (Fig. 10). The results with overlapping buffer systems at the same pH were closely similar but not identical, suggesting some specific buffer-ion effects. Under the present conditions the pH optimum for the oxidation of ethanol is 11 and for the reduction of acetaldehyde it is 6.5.

Substrate Inhibition.—Human liver-alcohol dehydrogenase is maximally active at an ethanol concentration of 1.7×10^{-2} M, but is inhibited at higher concentrations (Fig. 11). Inhibition is similar to but less pronounced than that observed with horse liver-alcohol dehydrogenase. The optimal ethanol concentration is the same at pH 7.0, 8.8, and 11.0, but higher concentrations of ethanol are less inhibitory at pH 7.0.

Substrate Specificity.—Human liver-alcohol dehydrogenase oxidizes a number of different alcohols. The rates of oxidation are compared in Table VI, in which ethanol is taken as a standard of reference, together with those of the horse-liver enzyme (Winer, 1958). In contrast to the enzyme from the horse, human alcohol dehydrogenase oxidizes methanol, 2-propanol, and ethylene glycol.

DISCUSSION

The purification procedure for alcohol dehydrogenase from human liver is similar to that employed for other enzymes from liver tissue. Many of these employ chloroform and ethanol to remove hemoglobin as initially suggested by Tsuchihashi (Keilin and Mann, 1941). In the present study hemoglobin is removed by chromatography rather than by the use of chloroform and ethanol. Repetitive chromatography, i.e., recycling on CM-cellulose, followed by DEAE-cellulose and again by CM-cellulose, is a much gentler procedure than precipitation by mixed solvents and hence more likely to give a high yield of active enzyme.[2] The concentrating effect of chromatography presents an additional advantage over the dilution effect incurred by precipitation procedures.

The most highly purified preparations of human liver-alcohol dehydrogenase obtained to date contains at least 2 g-atoms of zinc per mole of protein, the average value for five different preparations being 2.2 ± 0.2 g-atoms per mole of enzyme (Table II). These results suggest a value of 2 g-atoms of zinc per mole of enzyme; this tentative conclusion, however, requires further validation.

While zinc undergoes a 2-fold aggregation, the concentrations of all other metals decrease during purification.

[2] Further purification has been achieved by the use of electrophoresis on a Porath column (Blair and Vallee, unpublished).

TABLE VI
RELATIVE RATES OF OXIDATION OF ALIPHATIC ALCOHOLS BY HUMAN AND HORSE LIVER–ALCOHOL DEHYDROGENASES

	Human LADH	Horse LADH[a]
Conditions for assay		
pH/temp	8.8/23°	9.5/23.5°
(ADH)	1.6×10^{-6} M	1.44×10^{-3} M
(DPN^+)	1.66×10^{-3} M	1.2×10^{-4} M
(Alcohol)	1.16×10^{-3} M[b]	1.0×10^{-3} M
Substrate		
Methanol (0.5 M)	1.2	0
Ethanol	1.0[c]	1.0[c]
1-Propanol	1.4	1.1
2-Propanol	0.4	0
1-Butanol	1.7	1.6
Isoamyl alcohol	1.5	1.2
1-Hexanol	1.5	1.3
Ethylene glycol (1.6×10^{-2} M)	0.1	

[a] Winer (1958). [b] Except where indicated. In these cases the rate is compared to that on ethanol at the same concentration. [c] Set equal to one as a standard.

Calcium, in insignificant amount, is the only other metal present in the most highly purified preparation (Table III). Small and variable amounts of iron and aluminum are found in preparations which did not undergo rechromatography on CM-cellulose (Table II); the results of electrophoresis suggest that they are removed apparently during this final purification step (Table III).

Considerations governing the use of certain inhibitors of enzymatic activity to determine the functional significance of the metal atoms in metalloenzymes in conjunction with analytical data have been discussed (Vallee, 1955, 1960). Human liver–alcohol dehydrogenase is inhibited by metal-binding agents when they are present in the assay mixture. An analogous inhibition of other metallodehydrogenases by such agents has been demonstrated (Hoch *et al.*, 1958; Williams *et al.*, 1958; Vallee *et al.*, 1956; Vallee, 1960). Although the complexing agents employed here vary in molecular structure and configuration, all of them bind zinc ions though they are not specific in their capacity to form chelates with only this particular metal. Since zinc is the only metal present in the purified enzyme, however, the presumptive evidence is strong that the inhibition of activity must be owing to the interaction of the chelating agents with the zinc atoms (Table IV). The action of two of these, 1,10-phenanthroline and 8-hydroxyquinoline, has been investigated in greater detail. The effects of both depend only upon their concentration in the reaction mixture (Fig. 2). The inhibition, and hence the equilibrium in the assay mixture, is instantaneously reversible upon dilution.

Added metal ions successfully compete with the enzyme zinc for 1,10-phenanthroline, a circumstance which is demonstrated in two different ways. In the first case, Zn^{2+} together with 1,10-phenanthroline are added to the reaction mixture *prior to* the addition of the enzyme (Fig. 3). The effect upon the degree of inhibition is directly proportional to the amount of free Zn^{2+} present in the solution. Second, once the enzyme is inhibited by 1,10-phenanthroline this inhibition can be reversed by the addition of Zn^{2+} (Fig. 4). These functional consequences, together with the analytical results, meet the established operational criteria required to identify metalloenzymes in which

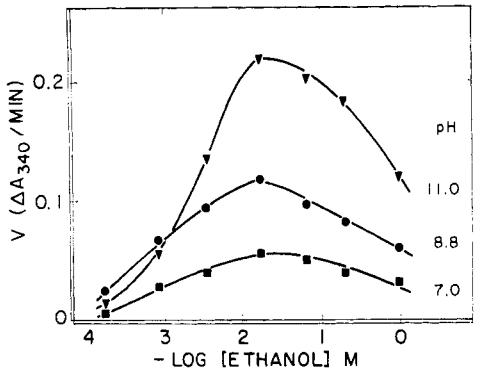

FIG. 11.—Effect of varying concentrations of ethanol upon the enzymatic activity of human liver–alcohol dehydrogenase at different *p*H values. DPN^+, 1.6×10^{-3} M; 23°. *p*H 11.0 (▼), 3.3×10^{-2} M glycine-NaOH; *p*H 8.8 (●), 3.3×10^{-2} M sodium pyrophosphate; *p*H 7.0 (■), 3.3×10^{-2} M sodium phosphate. The change in absorbance at 340 mμ/min is plotted against the negative logarithm of the ethanol concentration.

the metal serves an essential functional role while firmly incorporated in the active site (Vallee, 1960).

Electrophoresis in a phosphate buffer was performed at three *p*H values, 7, 7.4, and 8. In all three instances the active protein boundary moved toward the anode, suggesting that in these buffers the isoelectric point is above that of the horse enzyme, *p*H 6.8. At *p*H 7.4, however, the impurity moved toward the cathode. Its isoelectric point is therefore presumably between *p*H 7.0 and 7.4.

Sedimentation of a number of different preparations shows only a single boundary, suggesting that the heterogeneity revealed by electrophoresis is not owing to a difference in mass or frictional coefficient, but rather to a difference in charge. The value of $s^\circ_{20,w}$, 4.80 ± 0.01 S, is lower than that found for the horse enzyme, 5.11 S. The molecular weight of 87,000, however, is higher than that for the horse enzyme, 84,000. The time course of the molecular-weight determination, however, indicated that this sample was somewhat heterogeneous. The physicochemical criteria and enzymatic indices imply that the present enzyme preparations are not as yet completely homogeneous. However, present data indicate that relatively little effort is required to achieve a completely purified preparation. Such efforts are currently in progress.

Electrophoresis and sedimentation studies, taken together, imply, however, that the gross physical characteristics of this enzyme strongly resemble those of the horse enzyme.

The presence of two zinc atoms per mole of enzyme suggests that the human enzyme, like that of the horse, has two active centers per molecule. The results of spectral titration with DPNH lend further strength since one molecule of enzyme binds two moles of DPNH (Fig. 9). The characteristics of the human LADH-DPNH complex are qualitatively and quantitatively nearly identical to those of the horse enzyme (Fig. 8), including the isosbestic point.

Thus, in respect to the main structural features, these two mammaliam enzymes are very similar. Their functional characteristics differ significantly though many of the differences are in degree rather than in kind.

The patterns of the Michaelis constants for the coenzymes and substrates (Table V) are similar to those of the horse enzyme. However, those of the human enzyme are from two to ten times greater than those of the horse enzyme. The *p*H rate profiles for both

oxidation and reduction are similar to those of the horse enzyme (Theorell and Bonnichsen, 1951) (Fig. 10), as is the substrate inhibition induced by ethanol. Figure 11 shows that while the *p*H optimum at high substrate concentrations is *p*H 11, that at low concentration is *p*H 9.

The most striking difference between the two enzymes is in their substrate specificities (Table VI). While methanol is a good substrate for the human enzyme, the horse enzyme oxidizes it poorly, if at all. Human liver-alcohol dehydrogenase, therefore, has a wider substrate specificity.

This broader biological specificity of the alcohol dehydrogenase from human liver is of great significance in relation to the toxicity of alcohols such as methanol and ethylene glycol. Treatment of methanol toxicity in humans with ethanol has long been employed empirically. It was known that the oxidation products of methanol are the ultimate toxins and that ethanol acts competitively to prevent the oxidation of methanol. The failure of the horse enzyme to utilize methanol as a substrate, (Sund and Theorell, 1963; Winer, 1958), however, led to the conclusion that a simple competition between methanol and ethanol for the active site of alcohol dehydrogenase could not account for the protective effect of ethanol. The isolation of the human enzyme has permitted experimental demonstration that methanol is indeed a substrate and that ethanol serves to inhibit its oxidation. The present studies provide a basis for the observed clinical effectiveness of ethanol therapy.

Similarly ethylene glycol, an ingredient of many commercial antifreeze compounds, is also a substrate for human liver-alcohol dehydrogenase. This compound, while not itself toxic, is oxidized to the highly toxic oxalic acid. Ethanol prevents this oxidation both *in vitro* and *in vivo*. Furthermore, the toxicity of ethylene glycol can be abolished both in the monkey and in man by administration of ethanol in concentrations high enough to compete for the active site of human liver-alcohol dehydrogenase (Peterson *et al.*, 1953; J. E. Coleman, paper in preparation).

Finally, the isolation and purification of human liver-alcohol dehydrogenase provides an opportunity to examine for the first time the molecular basis of both normal and abnormal ethanol metabolism in man (Vallee *et al.*, 1957).

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