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Rat Liver Alcohol Dehydrogenase

PURIFICATION AND PROPERTIES

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Alcohol dehydrogenase (EC 1.1.1.1) from the rat liver supernatant fraction has been purified 200-fold and partially characterized. The isolation procedure involved ammonium sulphate fractionation, DEAE-Sephadex chromatography and gel filtration. The purified enzyme behaved as a homogeneous preparation as evaluated by cellulose acetate and polyacrylamide-gel disc electrophoresis. Sulphoethyl-Sephadex chromatography and immunoelectrophoresis with rabbit antiserum indicated the presence of a minor component. Rat liver alcohol dehydrogenase appears to contain 4mol of zinc/mol, has an estimated molecular weight of 65000 and consists of two subunits of similar molecular weight. Heavy-metal ions, thiol-blocking reagents, urea at concentrations below 8M, low pH (5.5) and chelating agents deactivate the enzyme but do not dissociate it into subunits. Deactivated enzyme could not be reactivated. The enzyme is strictly specific for NAD^+ and has a broad specificity for alcohols, which are bound at a hydrophobic site. Inhibition occurred with the enzyme equilibrated with Zn^{2+} at concentrations above 0.1mM.

Alcohol dehydrogenase (alcohol–NAD^+ oxidoreductase, EC 1.1.1.1) is a zinc-dependent metalloenzyme localized in the cytoplasm (Nyberg, Schuberth & Änggärd, 1953). It has been isolated and purified from yeast (Hayes & Velick, 1954), the fruitfly *Drosophila* (Jacobson & Pfuderer, 1970), horse liver (Bonnichsen & Wassen, 1948; Dalziel, 1961) and human liver (Wartburg, Bethune & Vallee, 1964).

Little information is available about rat liver alcohol dehydrogenase apart from its implication in steroid dehydrogenation (Okuda & Takigawa, 1970). Enzyme from this source has been studied in liver homogenates (Buettner, 1965) and crude enzyme preparations (Reynier, 1969), but attempts to obtain highly purified preparations have not been successful. The present paper describes the purification and some of the physicochemical properties of the enzyme.

EXPERIMENTAL

Purification of rat liver alcohol dehydrogenase

Precautions. All laboratory glassware was soaked overnight in 10M-HNO_3; plastic and stainless-steel utensils were soaked in 0.1% EDTA. Subsequently, all were rinsed with double-distilled water. Reagent and buffer solutions were made in double-distilled water. To avoid frothing, enzyme was precipitated from solution by slow dialysis overnight against solid $(NH_4)_2SO_4$. Only enzyme-grade $(NH_4)_2SO_4$ that gave a neutral reaction in aqueous solution was used. All procedures were carried out at 0–4°C unless otherwise stated.

Preparation of high-speed liver supernatant fraction. The details that follow are given for a typical preparation and correspond to the information presented in Table 1. Sprague–Dawley rats (12) of 150–250g body wt. were used. Each rat was anaesthetized with ether, the abdominal and thoracic cavities were opened and as much blood as possible was withdrawn from the heart. The liver was perfused by introduction of 40–80ml of cold 0.85% NaCl into the aorta, excess of fluid escaping from the severed vena cava. Then, the liver was removed, blotted with ashless filter paper, minced and homogenized in 9vol. of 0.32M-sucrose in 10mM-tris–HCl buffer, pH7.5, in a Potter–Elvehjem homogenizer with a Teflon pestle. The pooled homogenates (1050ml) were centrifuged at 23500*g* for 1h in a refrigerated Sorvall centrifuge, model RC2-B. The high-speed supernatant fraction (930ml) was used for enzyme purification.

Ammonium sulphate fractionation. The high-speed supernatant fraction was 35% saturated with $(NH_4)_2SO_4$ (209g/l). After complete equilibration, the suspension was centrifuged for 30min at 23500*g* in a Sorvall refrigerated centrifuge and the precipitate was discarded. The concentration of $(NH_4)_2SO_4$ was then increased to 60% (addition of 164g/l). The suspension was centrifuged for 30min at 23500*g* as described above. The grey pellet recovered was redissolved in 70ml of 40mM-tris–HCl buffer, pH7.6, and dialysed against 4 litres of the same buffer for 24–48h. The non-diffusible material was removed by centrifugation.

DEAE-Sephadex chromatography. The dialysed, clear

35–60%-saturated-$(NH_4)_2SO_4$ fraction (101 ml) was applied to a column (36 cm × 6.8 cm) of DEAE-Sephadex A-50 equilibrated and eluted with 40 mM-tris–HCl buffer, pH 7.6. Fractions of high specific activity appeared after 590 ml had been eluted. These fractions were pooled (149 ml) and precipitated by 62% saturation with $(NH_4)_2SO_4$ (406 g/l).

Gel filtration. The enzyme precipitated by 62%-saturated $(NH_4)_2SO_4$ was redissolved in 10 ml of 50 mM-tris–HCl buffer, pH 7.5, and clarified by centrifugation. The enzyme solution (10 ml) was applied to a column (50 cm × 5.2 cm) of Sephadex G-100 equilibrated and eluted with 50 mM-tris–HCl buffer, pH 7.5. Fractions of high specific activity appeared after 405 ml was collected. These were pooled (31 ml) and precipitated by 62% saturation with $(NH_4)_2SO_4$ (406 g/l). The enzyme was redissolved in 5 ml of the same buffer and gel-filtered once again through a column (54 cm × 5.9 cm) of Sephadex G-100 under similar conditions. Enzymic activity appearing after 490 ml was pooled (62 ml) and precipitated by 62% saturation with $(NH_4)_2SO_4$ (406 g/l).

Chromatography on sulphoethyl-Sephadex. The precipitated enzyme was dissolved in 1 ml of 10 mM-potassium phosphate buffer, pH 7.0, containing 20 mM-NaCl and was dialysed against 250 ml of the same buffer for 2 h. The non-diffusible material (1 ml) was applied to a column (18 cm × 3.2 cm) of sulphoethyl-Sephadex equilibrated with the same buffer. Then a linear gradient was developed by introducing 150 ml of 0.2 M-NaCl in 10 mM-potassium phosphate buffer, pH 7.0, into a mixing chamber containing 150 ml of the equilibration buffer. The enzyme was eluted between 0.15 M- and 0.17 M-NaCl. Enzyme appeared after 195 ml was collected and the pooled active fractions (31 ml) were precipitated by 62% saturation with $(NH_4)_2SO_4$ (406 g/l).

Other methods

Analytical disc electrophoresis. Disc electrophoresis in 7.5% (w/v) polyacrylamide gels was performed by the methods of Varon, Nomura & Shooter (1967) at pH 7.0, Clarke (1964) at pH 8.1 and Davis (1964) at pH 9.5, with omission of the separating and the sample gels. Samples of 0.2 ml in 0.4–0.6 M-sucrose were layered gently on top of the gel. A constant current of 4 mA/tube was applied for 2 h at room temperature. The gels were stained for at least 1 h in 0.1% Amido Schwartz in 7% (v/v) acetic acid. Enzyme activity was located by incubating the gels in a medium containing 1.5 ml of Nitro Blue Tetrazolium (1 mg/ml), 0.15 ml of phenazine methosulphate (1 mg/ml), 5 mg of NAD^+ added as solid, 2 ml of 0.2 M-glycine–NaOH buffer, pH 9.5, and 0.2 ml of 96% (v/v) ethanol for 30 min at 37°C.

Immunoelectrophoresis in agar. Immunoelectrophoresis was performed at 150 V in 0.1 M-sodium barbiturate buffer, pH 8.2 and I 0.1. The slides were prepared with purified agar (1%) and NaN_3 (0.01%) in the same buffer. Immunoelectrophoretic patterns developed within 1 day in a moist chamber in the refrigerator.

Electrophoresis on cellulose acetate strips. Electrophoresis on cellulose acetate strips was carried out at 300 V in 50 mM-tris–glycine buffer, pH 8.1, for 1 h.

Measurement of enzyme activity. Alcohol dehydrogenase activity was measured by the method of Bonnichsen (1963) in a Perkin–Elmer model 202 recording spectrophotometer at 340 nm. Final NAD^+ and ethanol concentrations were 2.0 mM and 0.54 mM respectively. The enzyme was assayed in 75 mM-sodium pyrophosphate–22 mM-glycine buffer, pH 8.8, at 25°C in a total volume of 1.0 ml. The control cuvette contained all reaction components except ethanol. Enzyme activity is expressed as μmol of NAD^+ reduced/min.

Measurement of zinc. Samples containing 0.5–5 μg of zinc were digested with 1.0 ml of HNO_3 (70%, w/w) followed by 1.0 ml of $HClO_4$ (70%, w/w) and evaporated to dryness. The residue was dissolved in 1–5 ml of deionized water and the zinc concentration was determined in a Perkin–Elmer model 303 atomic-absorption spectrometer with a hollow cathode lamp at 213.8 nm. The flame was provided by a mixture of acetylene and compressed air. Column eluates were aspirated directly. Solutions of $ZnCl_2$ of known concentration were used to construct standard curves. For column eluates that contained NaCl, standard solutions were prepared in 0.1 M-NaCl.

Measurement of proteins. Protein was usually determined by the procedure of Lowry, Rosebrough, Farr & Randall (1951). Samples of 0.1 ml were precipitated with 10 vol. of cold 5% (w/v) trichloroacetic acid and the protein was redissolved in 1–3 ml of 0.1 M-NaOH. Crystalline bovine serum albumin (fraction V, Sigma Chemical Co., St Louis, Mo., U.S.A.) stored at low temperature in the presence of a dessicating agent was used for construction of standard curves. In enzyme fractions that were clear and colourless, the protein concentration was determined spectrophotometrically from E_{280} and E_{260} values (Warburg & Christian, 1942).

Determination of molecular weight. Molecular weight of the enzyme was determined by gel filtration by the method of Andrews (1965). Sephadex G-150 and G-100 columns (50 cm × 1.7 cm) equilibrated and eluted with 50 mM-tris–HCl buffer, pH 7.5, were used. The following crystalline proteins served as molecular-weight markers: bovine serum albumin (mol.wt. 66000), egg albumin (mol.wt. 96000) (Neurath, 1963); cytochrome *c* (mol.wt. 11700), sperm-whale myoglobin (mol.wt. 17200), lysozyme (mol.wt. 14300) (Eck & Dayhoff, 1966); malate dehydrogenase (mol.wt. 72000; Wolfenstein, Englard & Listowsky, 1969); horse liver alcohol dehydrogenase (mol.wt. 80000; Drum, Li & Vallee, 1969); pepsin (mol.wt. 35000; Bovey & Yanari, 1960). Horse liver alcohol dehydrogenase and malate dehydrogenase activities were assayed by the methods of Vallee & Hoch (1955) and Hohorst (1963) respectively. The extinctions of cytochrome *c* and myoglobin at 410 nm, Blue Dextran at 260 nm and the remaining proteins at 280 nm were read in a Beckman model DU spectrophotometer.

To study possible changes in molecular weight due to aggregation or dissociation, columns (50 cm × 1.7 cm) of Sephadex G-150 were equilibrated and eluted with 10 mM-EDTA in 50 mM-tris–HCl buffer, pH 7.5, 50 mM-tris–HCl buffer, pH 8.9, or 50 mM-sodium acetate buffer, pH 5.4. Bovine serum albumin and egg albumin were used as molecular-weight markers.

Sodium dodecyl sulphate–polyacrylamide gels. Samples containing 25–50 μg of protein were applied on the 5% (w/v) polyacrylamide-gel system of Dunker & Rueckert (1969) without iodoacetamide treatment. The

relative migration of bands was based on that of Bromophenol Blue (0.1%). A constant current of 6 mA/tube was applied for 2 h. The gels were left for 24 h in 20% (w/v) sulphosalicylic acid and then were stained with Amido Schwartz.

Preparation of antibodies in rabbits. Antibodies against partially purified alcohol dehydrogenase (after chromatography on DEAE-Sephadex) were prepared by thoroughly mixing enzyme solution containing 1 mg of protein/ml with an equal volume of Freund's complete adjuvant and injecting 2.0 ml of the emulsion/animal subcutaneously (0.25 ml in each hind paw, and three injections of 0.5 ml in the neck region). After 2 weeks a booster injection of the same enzyme preparation in 25 mM-tris–HCl in 0.85% NaCl was injected into each animal. Then 2 weeks later the rabbits were exsanguinated by heart puncture. The sera were divided into small portions and stored at −20°C.

Plates for immunodiffusion contained agar (0.1%) and NaN_3 (0.01%) in 34 mM-sodium barbiturate–103 mM-NaCl buffer, pH 7.4 and I 0.15.

RESULTS

Purification of rat liver alcohol dehydrogenase

Rat liver alcohol dehydrogenase could not be purified by conventional methods used for other alcohol dehydrogenases because of substantial deactivation both in our laboratory and elsewhere (Reynier, 1969). Hence mild and rapid conditions were used in the purification procedure. A 35–60% saturation with ammonium sulphate concentrated 92% of the enzyme (Table 1). Exhaustive dialysis against 50 mM-tris–HCl buffer, pH 7.6, precipitated some extraneous proteins. During DEAE-Sephadex chromatography (Fig. 1) most proteins, including haemoglobin, were adsorbed on the resin whereas enzyme was eluted. A 10–15-fold purification was achieved at this step. Two successive gel filtrations on Sephadex G-100 efficiently separated the enzyme from non-enzyme protein (Fig. 2). Good resolution and fair yields (10–40%) were obtained provided that flow through the columns was rapid and the eluted enzyme was concentrated as soon as possible by precipitation with ammonium sulphate. At this stage maximal specific activity of the enzyme was obtained, 1–4 μmol of NAD^+ reduced/min per mg of protein. Ion-exchange chromatography of the enzyme on sulphoethyl-Sephadex caused partial deactivation and an inactive zinc-containing protein was eluted first, followed by the enzyme. Evidently the necessity of using a low-ionic-strength buffer to adsorb the enzyme on the resin resulted in partial deactivation of the enzyme. In fact, during chromatography some of the zinc was lost from the enzyme and zinc/protein ratios fell from 2.94 to 1.07 μg of zinc/mg of protein (Table 1).

Analytical disc electrophoresis. On disc electrophoresis at pH 7.0 and 8.1 and after staining for

Table 1. *Purification of rat liver alcohol dehydrogenase*

Experimental details are given in the Experimental section.

Fraction	Volume (ml)	Activity (nmol/min per ml)	Zinc (μg/ml)	Protein (mg/ml)	Zinc/protein ratio (μg/mg)	Specific activity (nmol/min per mg of protein)	Yield (%)	Purification (fold)
23000 *g* supernatant solution	930	43.2	1.66	6.9	0.24	6.3	100	1
35–60%-saturated $(NH_4)_2SO_4$ fraction	101	365.0	1.27	32.8	0.04	11.1	92	2
DEAE-Sephadex fraction	149	192.2	1.01	1.08	0.94	178.0	71	30
Sephadex G-100 fraction	31	746.0	1.19	1.60	0.74	466.3	58	74
Sephadex G-100 fraction	62	215.0	0.53	0.18	2.94	1194	33	190
Sulphoethyl-Sephadex fraction	31	158.1	0.15	0.14	1.07	1124	12	179

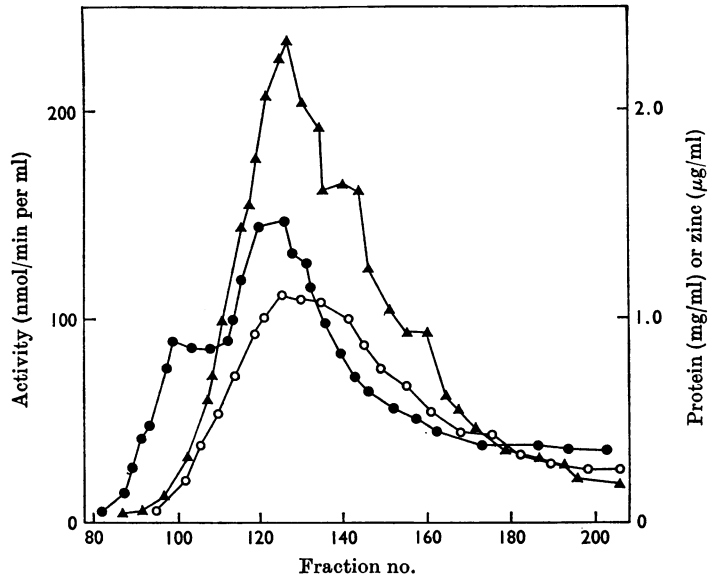


Fig. 1. Elution pattern of rat liver alcohol dehydrogenase of 36–60%-saturated-$(NH_4)_2SO_4$ fraction on a DEAE-Sephadex column (36 cm × 6.8 cm) equilibrated and eluted with 40 mM-tris–HCl buffer, pH 7.6. Fractions of volume 5.0 ml were collected. Zinc in the eluate (○) was determined directly by atomic-absorption spectrometry. Protein (●) was calculated from E_{280} and E_{260} values. Enzyme activity (▲) was assayed as described in the Experimental section.

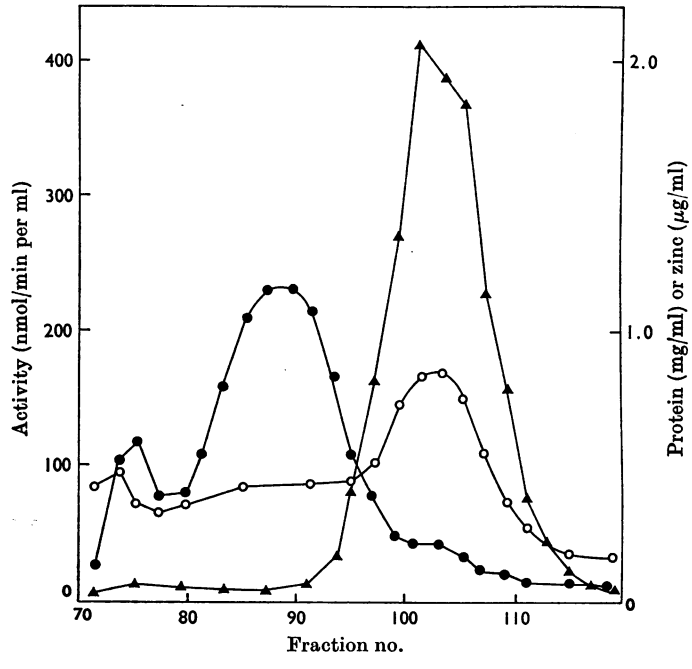
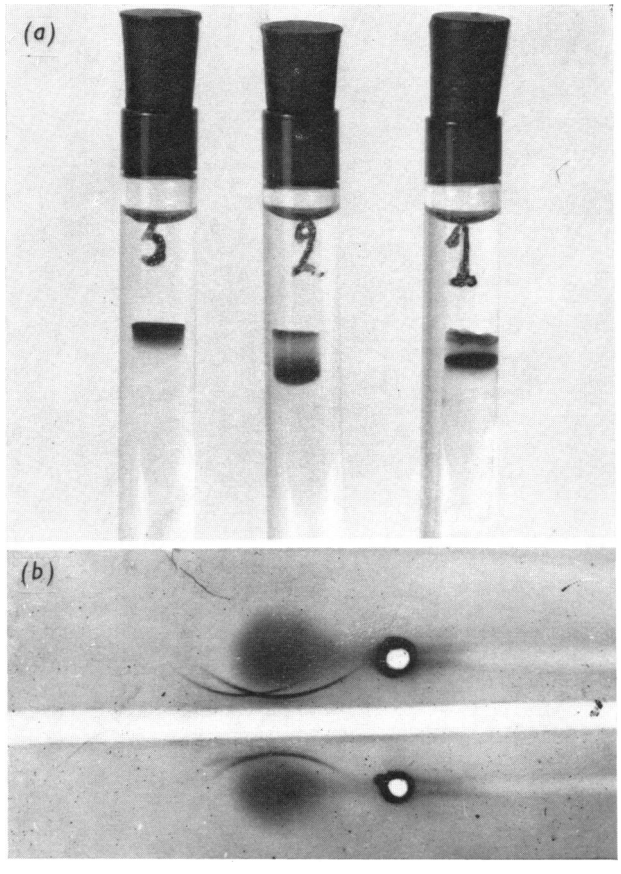


Fig. 2. Gel filtration of rat liver alcohol dehydrogenase from Sephadex G-100 fractionation of a second Sephadex G-100 column (54 cm × 5.9 cm) equilibrated and eluted with 50 mM-tris–HCl buffer, pH 7.5. Fractions of volume 5.0 ml were collected. Zinc (○), protein (●) and enzyme activity (▲) were determined as indicated in Fig. 1.



EXPLANATION OF PLATE 1

(*a*) Polyacrylamide-gel disc electrophoresis of rat liver alcohol dehydrogenase from the second Sephadex G-100 fraction. Tubes 1, 2 and 3 were run at pH 7.0, 8.1, and 9.5 respectively. The samples applied were 50 μg. The gels were stained with Amido Schwartz as described in the Experimental section. (*b*) Immunoelectrophoresis of different rat liver alcohol dehydrogenase preparations (second Sephadex G-100 fraction) at 1 mg/ml concentration with rabbit anti-serum against the partially purified enzyme. The dark spots behind the arcs were developed by the enzyme staining technique described in the Experimental section.

protein, the purified enzyme showed a single band (Plate 1*a*). The enzyme failed to enter the polyacrylamide gel at pH 9.5. Staining for enzyme activity often revealed the presence of two diffuse bands, the major one being identical with the protein band. The second activity band was not associated with detectable protein.

Immunoelectrophoresis. Immunoelectrophoresis of dialysed 35–60%-saturated-ammonium sulphate preparation of the enzyme indicated the presence of six or seven distinct antigens. The purified enzyme showed one major and one minor arc when enzyme preparations of 1mg or more of protein/ml were applied (Plate 1*b*). The major arc was identified as the enzyme protein by staining for activity before the addition of the antiserum. The nature of the second arc is unknown. It might be a contaminant since the two antigens did not cross-react.

Electrophoresis on cellulose acetate. Electrophoresis of the purified enzyme gave one activity or one protein band depending on the staining technique.

Physical properties of rat liver alcohol dehydrogenase

Molecular weight and subunit structure. The estimated molecular weight of rat liver alcohol dehydrogenase was 65000 ± 5000 when determined by gel filtration on calibrated Sephadex (G-150 and G 100) columns of different size, utilized to exclude adsorption or anomalous shape and size effects (Fig. 3). The enzyme immediately followed bovine serum albumin and had a similar elution volume. In good agreement with gel-exclusion results, sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of the enzyme indicated a molecular weight of 56000–60000 for the enzyme and 37000–41000 for the subunit (Fig. 4). Evidently the enzyme consists of two subunits of similar molecular weight. An aggregate of 100000–110000 molecular weight was also detected. It is presumed that the aggregate corresponds to a dimeric species. Ultrafiltration of the purified enzyme through Sartorius membrane filter SM11736 retained 40% of the enzyme, providing further evidence for a molecular weight close to 70000. The 35–60%-saturated-ammonium sulphate preparation of the enzyme did not enter 7.5% polyacrylamide gels at pH 7.0, 8.1 or 9.5, a finding that suggests the presence of high-molecular-weight aggregates of the enzyme or its association with other proteins.

Association–dissociation studies. Gel filtration of enzyme through Sephadex columns equilibrated with EDTA or at low pH (5.4) demonstrated no change in elution volume of the enzyme protein. Molecular sieving at high pH (8.9) failed to show the presence of aggregates of larger molecular weight.

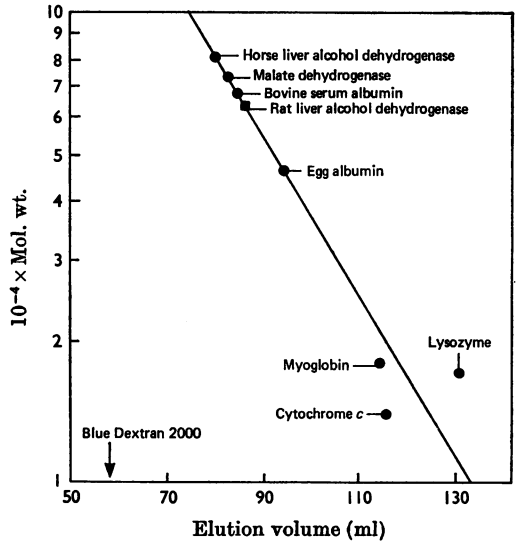


Fig. 3. Molecular-weight determination of rat liver alcohol dehydrogenase from second Sephadex G-100 fraction on a Sephadex G-150 column (50cm × 1.7cm). The samples applied contained 1–7mg, and fractions of 2.0ml were collected. ●, Proteins and Blue Dextran used as molecular-weight markers (for molecular weights see the Experimental section); ■, rat liver alcohol dehydrogenase.

Catalytic properties of rat liver alcohol dehydrogenase

Substrate and coenzyme requirements. The apparent K_m values of the enzyme for ethanol and NAD^+ determined at pH 8.8 in 75mM-sodium pyrophosphate–22mM-glycine buffer at 25°C were 4×10^{-2}M and 3×10^{-4}M respectively. The enzyme showed activity with a wide variety of aliphatic alcohols (Table 2), pentan-1-ol being oxidized at the fastest rate. The absolute specific activity of this enzyme preparation with pentan-1-ol was 7.0 μmol/min per mg of protein.

Inhibition by chelating agents. A study of the inhibition of the enzyme by chelating agents incorporated into the assay medium revealed a subtle difference in their behaviour towards the enzyme (Table 3). Sodium azide, αα′-bipyridyl, nitrilotriacetic acid and EDTA caused little inhibition even at high concentrations. On the other hand, 8-hydroxyquinoline and 1,10-phenanthroline caused drastic inhibition.

Prolonged treatment of the enzyme with EDTA led to inhibition which varied with the concentration of the chelating agent. Dialysis for 24h against 0.1mM-, 1.0mM- or 10mM-EDTA in 50mM-tris–HCl buffer, pH 7.6, resulted in 7, 8 and 76% inhibition of activity respectively. Concomitantly, the enzyme lost 32, 44 and 77% of its zinc. Addition

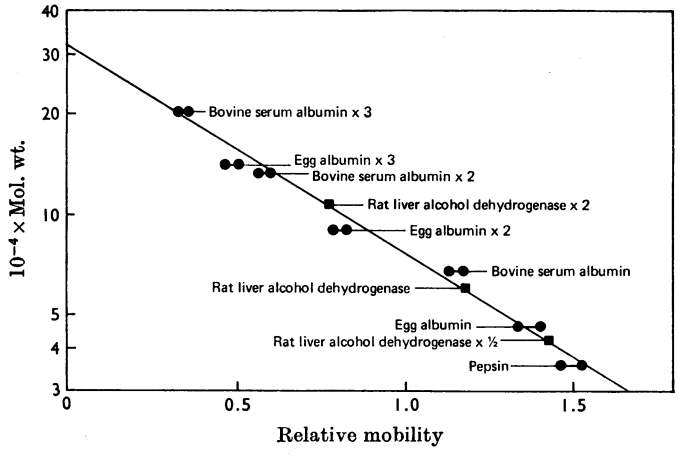


Fig. 4. Molecular-weight determination of rat liver alcohol dehydrogenase from the second Sephadex G-100 fraction by sodium dodecyl sulphate—polyacrylamide-gel electrophoresis. ●, Proteins used for molecular-weight calibration (for molecular weight, see the Experimental section): ■, rat liver alcohol dehydrogenase; ×1/2, ×2 and ×3 refer to the subunit, dimer and trimer species respectively.

Table 2. *Substrate specificity of rat liver alcohol dehydrogenase*

Rates of oxidation of the alcohols were compared with that of pentan-1-ol (7 μmol/min per mg of protein). Assay conditions were as described in the Experimental section. Some of the alcohols could not be used at 0.54 M because of their limited solubility in the medium and their final concentration appears in parentheses. Enzyme (0.1 mg of protein/ml, final concentration) from the second Sephadex G-100 fraction was used.

Substrate	Activity (%)	Substrate	Activity (%)
Methanol	4	Propan-2-ol	8
Ethanol	17	Pentan-2-ol (507 mM)	19
Propan-1-ol	27	2-Methylpropan-2-ol	2
Butan-1-ol	33	2-Methylbutan-2-ol	None
Pentan-1-ol (243 mM)	100	Propane-1,2-diol	12
Hexan-1-ol (61 mM)	34	Glycerol	None
Heptan-1-ol (9 mM)	29	DL-Lactate	None
Octan-1-ol (5 mM)	12		
2-Methylpropan-1-ol	18		
3-Methylbutan-1-ol (303 mM)	17		

of Zn^{2+} or dialysis against 10 μM-Zn^{2+} failed to restore activity.

Effect of some metabolites. The effect of some common metabolic intermediates on the oxidation of ethanol by the enzyme was tested (Table 4). Pyruvate, oxaloacetate and L-malate caused 100% inhibition. In the presence of citrate or succinate, some activation was observed. This activation does not seem to be due to chelation of zinc, as no similar effect was observed in the presence of EDTA (Table 3).

Stability of rat liver alcohol dehydrogenase

Enzyme stability and thiol-blocking reagents. The enzyme was stable for at least 3 months in 60%-saturated ammonium sulphate in 50 mM-tris–HCl buffer, pH 7.6, at 4°C. Thiols were not needed to protect the enzyme provided that frothing, surface-active denaturing agents and contamination by heavy-metal ions were avoided. Dithiothreitol partially protected the enzyme in the presence of these factors. Dialysis of the enzyme (1 mg of protein/ml) for 24 h against *N*-ethylmaleimide, iodoacetamide, iodoacetate and *p*-chloromercuribenzoate each at 1 mM in 50 mM-tris–HCl buffer, pH 7.6, caused 91, 90, 97 and 100% inactivation respectively. These findings demonstrate the importance of cysteine groups in the enzyme.

Temperature, pH and urea. The enzyme was relatively stable towards heat. After 5 min at 63°C there was 50% deactivation.

Table 3. *Effect of metal-chelating agents on rat liver alcohol dehydrogenase*

The assay conditions were as described in the Experimental section except that the assay medium contained the specified concentration of metal-binding agent. v_i and v_0 are the initial velocities of the reaction determined in the presence and absence of the inhibitor respectively. Enzyme from the second Sephadex G-100 fraction (0.1 mg/ml, final concentration) was used.

Complexing agent	Concn. (mM)	v_i/v_0
NaN_3	0.1	0.93
	1.0	0.89
	10.0	0.79
αα′-Bipyridyl	0.1	1.00
	1.0	0.99
	10.0	0.79
EDTA	0.1	1.01
	1.0	1.00
	10.0	0.95
Nitrilotriacetic acid	0.002	1.00
	0.01	1.00
	0.06	0.98
8-Hydroxyquinoline	0.05	0.90
	0.1	0.89
	1.0	0.51
1,10-Phenanthroline	0.01	0.89
	0.1	0.81
	2.0	0.56

The pH optimum of the enzyme was 8.7–8.9 in tris, pyrophosphate and glycine–NaOH buffers. Dialysis of the enzyme overnight against buffers of different pH values indicated that below pH 5.5 the enzyme was completely deactivated (Fig. 5); maximum stability was in the pH range 7.0–8.5. Lowering of the pH of an enzyme solution to 5.0 followed by rapid neutralization in the presence or absence of Zn^{2+} failed to reactivate the enzyme.

Dialysis of the enzyme (1 mg of protein/ml) for 24h at 4°C against urea at 1–3 M in 50 mM-tris–HCl buffer, pH 7.6, caused less than 30% inhibition. However, the enzyme was completely deactivated when the urea concentration was raised from 3 to 5 M. Immunodiffusion studies showed that enzyme treated with 1 M-, 3 M- or 5 M-urea as well as acid pH (5.0) reacted with rabbit antiserum. No precipitation band appeared in the well containing enzyme treated with 8 M-urea.

Zinc content and the effect of zinc on rat liver alcohol dehydrogenase

Zinc content. Zinc content was calculated from zinc/protein ratios of the most active preparations on the assumption of a molecular weight of 65000 for the enzyme. In the preparation cited in Table 1, 2.94 μg of zinc/mg of protein or 2.9 mol of zinc/mol was present in the enzyme. As the specific activity of the purified enzyme varied, so did the zinc content. Estimates of 1–4 mol of zinc/mol of enzyme were obtained. During sulphoethyl-Sephadex chromatography, the zinc content fell to 1 mol/mol. Presumably up to 4 zinc atoms are present in the fully active enzyme molecule; during isolation some of these are lost.

Table 4. *Effect of some metabolic intermediates on the oxidation of ethanol by rat liver alcohol dehydrogenase*

Assay conditions were as described in the Experimental section except that the assay medium was made 1 mM with respect to the intermediate added. Enzyme (0.1 mg/ml final concentration) from the second Sephadex G-100 fraction was used.

Intermediate added	Activity (%)
None	100
Pyruvate	None
L-Lactate	99
Acetate	100
Oxaloacetate	None
Citrate	116
Succinate	125
L-Malate	None

Addition of Zn^{2+}. Purified enzyme supplemented with Zn^{2+} was inhibited 40% at 0.4 mM-Zn^{2+} (final concentration) compared with a control to which double-distilled water was added (Fig. 6). The chloride, sulphate and acetate salts of the metal had the same effect, indicating that inhibition was due to Zn^{2+} and not to the effect of the anion.

DISCUSSION

During purification of rat liver alcohol dehydrogenase, fractions containing high enzymic activity from ammonium sulphate fractionation, gel filtration, and chromatography on DEAE-Sephadex and sulphoethyl-Sephadex all contained high concentrations of zinc (Figs. 1 and 2). In the final preparations, zinc/protein ratios were increased 10–20-fold compared with those of the original liver fraction (Table 1). Also, removal of zinc by metal-chelating agents caused inhibition of enzyme activity. When taken together, these findings suggest that rat liver dehydrogenase is a zinc metalloenzyme and render unlikely the fortuitous association of this metal with the enzyme protein.

Table 3 shows that of the chelating agents tested in alkaline medium only 8-hydroxyquinoline and 1,10-phenanthroline inhibited the enzyme. As both of these compounds are known competitive inhibitors of NAD^+ in the reaction mediated by alcohol dehydrogenase (Anderson, Reynolds & Anderson, 1966), the observed inhibition may be due to this mechanism of action. Under the conditions used, it appears that other chelating agents

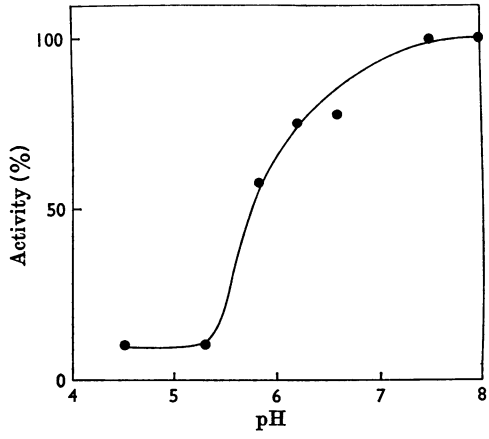


Fig. 5. Stability of rat liver alcohol dehydrogenase from the second Sephadex G-100 fraction at different pH values. Enzyme (1 mg/ml) dialysed overnight against 67 mM-Sorensen phosphate buffers at 4°C was assayed as described in the Experimental section.

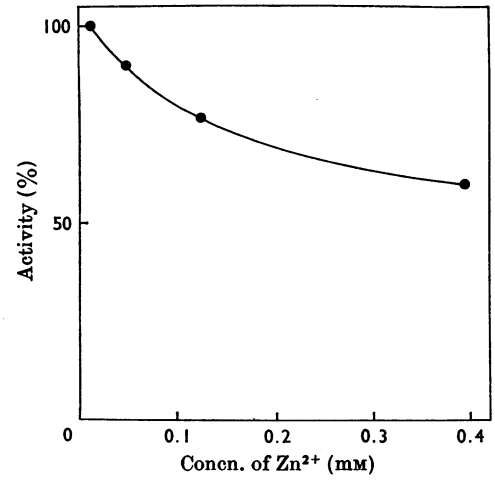


Fig. 6. Effect of Zn^{2+} on rat liver alcohol dehydrogenase from the second Sephadex G-100 fraction. Zinc acetate solutions (pH adjusted to 7.6) were added to 0.1 ml of enzyme (1 mg/ml) in 50 mM-tris–HCl buffer, pH 7.6. After 2 h equilibration, enzyme activity was assayed as described in the Experimental section. The final Zn^{2+} concentration at each point was determined by atomic-absorption spectrometry. The first point represents enzyme solution to which no Zn^{2+} was added.

cannot complex and remove zinc, or alternatively, that any zinc so removed has no detectable catalytic function. Hence differences in response to short-term and long-term inhibition by chelating agents might reflect differences in the nature of zinc atoms in rat liver alcohol dehydrogenase (Drum *et al.* 1969).

Equilibration of the enzyme with Zn^{2+} caused inhibition. A similar effect has been reported for another zinc metalloenzyme, alkaline phosphatase from *Escherichia coli* (Reynolds & Schlesinger, 1969) in the presence of Zn^{2+} exceeding 10 μM. Under these circumstances the enzyme has been shown to form a tetramer containing four times as much zinc as the monomer but possessing much less enzymic activity. However, the mechanism by which Zn^{2+} inhibits rat liver alcohol dehydrogenase may differ, as we have not been able to detect aggregates of larger molecular weight except on sodium dodecyl sulphate–polyacrylamide-gel electrophoresis.

Gel exclusion studies of the molecular weight of rat liver alcohol dehydrogenase have consistently shown that it is smaller than the enzyme isolated from horse liver (Fig. 6) and has a molecular weight similar to that of bovine serum albumin. Estimates of molecular weight for the undissociated enzyme obtained from sodium dodecyl sulphate–polyacrylamide-gel electrophoresis agreed with the above. Further, alcohol dehydrogenase of the fruitfly *Drosophila* has physical properties similar to those of the rat liver enzyme (Sober & Ursprung, 1968) and has been reported to have a molecular weight of 60000 (Jacobson & Pfuderer, 1970). Nevertheless, some evidence presented here suggests a higher molecular weight for the enzyme isolated from rat liver, foremost being the detection of a subunit of molecular weight 37000–41000 (Fig. 4). In addition, the finding of 2.9 mol of zinc/mol of enzyme of estimated molecular weight 65000 (Table 1) would not exclude the possibility of an enzyme of molecular weight of about 80000 containing 4 zinc atoms/mol, similar to the characteristics of the enzyme isolated from horse liver. A point of interest is comparison of the absolute specific activity of the enzymes from horse and rat liver. Winer (1958) has reported the value of 160 mol/litre per min per mol of horse liver alcohol dehydrogenase (mol.wt. 84000). Rat liver enzyme activity, when converted into the same units, yields the far higher value of 459 mol/litre per min per mol of enzyme (mol.wt. 65000), a difference greater than can be accounted for by the present uncertainty of the value to be assigned for the molecular weight. More precise information is required before either the molecular weight or the zinc stoicheiometry of this enzyme can be established. One factor contributing to the variability of the results presented here is loss of some zinc atoms during the process of purification, for according to calculations 4–5% of liver zinc should be associated with this enzyme, whereas experimental values are in the range 1–3%.

The very high apparent K_m for ethanol of rat liver alcohol dehydrogenase suggests that this is not the major physiological substrate (Okuda & Takigawa, 1970). Recently, however, Krebs & Perkins (1970) have demonstrated that fermentation in the intestine can account for production of ethanol and liver alcohol dehydrogenase for ethanol oxidation. An analogous search for C_4–C_6 aliphatic alcohols, that show maximal activity with the rat liver enzyme, might reveal their availability as naturally occurring substrates.

Substrate specificity studies of alcohol dehydrogenase isolated from different sources, while showing some specific differences, have demonstrated the basic similarity of a hydrophobic site for binding alcohols (Wartburg *et al.* 1964; Dickinson & Dalziel, 1967; Winer, 1958; Sober & Ursprung, 1968). Rat liver alcohol dehydrogenase also possesses this characteristic in common with the enzymes from other sources.

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