Holbrook, J. J., & Stinson, R. A. (1973) *Biochem. J. 131*, 739-748.
Holbrook, J. J., Liljas, A., Steindel, S. J., & Rossmann, M. G. (1975) *Enzymes (3rd Ed.) 11*, 191-268.
Lodola, A., Parker, D. M., Jeck, R., & Holbrook, J. J. (1978) *Biochem. J. 173*, 597-605.
Pogolotti, A., Jr., & Rupley, J. A. (1973) *Biochem. Biophys. Res. Commun. 55*, 1214-1219.
Schmid, F., Hinz, H. J., & Jaenicke, R. (1976) *Biochemistry 15*, 3052-3059.
Stamato, F. M. L. G., Longo, E., Ferreira, R., & Tapia, O. (1986) *J. Theor. Biol. 118*, 45-59.
Stinson, R. A., & Holbrook, J. J. (1973) *Biochem. J. 131*, 719-728.
Whitaker, J. R., Yates, D. W., Bennett, N. G., Holbrook, J. J., & Gutfreund, H. (1974) *Biochem. J. 139*, 677-697.
White, J. L., Hackert, M. L., Buehner, M., Adams, M. J., Ford, G. C., Lentz, P. J., Jr., Smiley, I. E., Steindel, S. J., & Rossmann, M. G. (1976) *J. Mol. Biol. 102*, 759-779.
Winter, G., Fersht, A. R., Wilkinson, A. J., Zoller, M., & Smith, M. (1982) *Nature (London) 299*, 756-758.
Wirz, B., Suter, F., & Zuber, H. (1983) *Hoppe-Seyler's Z. Physiol. Chem. 364*, 893-909.

Purification and Characterization of Human Liver Sorbitol Dehydrogenase†

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ABSTRACT: Sorbitol dehydrogenase from human liver was purified to homogeneity by affinity chromatography on immobilized triazine dyes, conventional cation-exchange chromatography, and high-performance liquid chromatography. The major form is a tetrameric, NAD-specific enzyme containing one zinc atom per subunit. Human liver sorbitol dehydrogenase oxidizes neither ethanol nor other primary alcohols. It catalyzes the oxidation of a secondary alcohol group of polyol substrates such as sorbitol, xylitol, or L-threitol. However, the substrate specificity of human liver sorbitol dehydrogenase is broader than that of the liver enzymes of other sources. The present report describes the stereospecific oxidation of (2*R*,3*R*)-2,3-butanediol, indicating a more general function of sorbitol dehydrogenase in the metabolism of secondary alcohols. Thus, the enzyme complements the substrate specificities covered by the three classes of human liver alcohol dehydrogenase.

The most complex pattern of alcohol dehydrogenase isozymes has been established for human liver (Vallee & Bazzone, 1983). Multiple forms include three classes (about 60% residue identity), nonallelic (about 90% residue identity) and allelic (more than 95% residue identity) class I homodimers as well as hybridized heterodimers between the individual subunits of class I isozymes (Jörnvall et al., 1987a). Primary structures of all class I isozymes of alcohol dehydrogenase from human liver have been determined (Jörnvall et al., 1987b) as well as those of the other two classes (Höög et al., 1987; Kaiser et al., 1988). The alcohol dehydrogenase family includes yet another enzyme, sorbitol dehydrogenase (L-iditol:NAD^+ 5-oxidoreductase, EC 1.1.1.14), since the primary structure of the corresponding enzyme from sheep liver demonstrated its sequence homology with alcohol dehydrogenases (Jeffery et al., 1981; Jörnvall et al., 1981). Despite the structural and mechanistic similarities, sorbitol dehydrogenase differs distinctly from the mammalian alcohol dehydrogenase in regard to the following: (i) it is a tetramer rather than a dimer; (ii) it contains one instead of two zinc atoms per subunit (Jeffery et al., 1984a); (iii) on the basis of model studies (Eklund et al., 1985), a glutamic acid residue is thought to replace one of the typical cysteine ligands of the catalytic zinc atom in alcohol dehydrogenase. Though sorbitol dehydrogenase activity was detected in many other species and tissues (Gerlach & Hiby, 1974), including human brain, lens, erythrocytes, and liver (O'Brien et al., 1983; Jedziniak et al., 1981; Barretto et al., 1985; Nealon & Rej, 1983), to date, the enzyme from sheep liver remains the only one for which structural information exists. Therefore, the present study describes the isolation and characterization of sorbitol dehydrogenase from human liver tissue. This not only will allow for an extensive and rigorous structure/function comparison within this family of enzymes from one source but also will contribute to reveal the metabolic role of sorbitol dehydrogenase, which has remained largely enigmatic. Furthermore, sorbitol dehydrogenase has previously attracted considerable interest, since its enzymatic activity has been linked to formation of cataracts and neuro-, retino-, and nephropathies (Gabbay, 1973), circumstances which have been adduced to explain the pathogenesis of diabetic complications (Greene et al., 1987).

This report demonstrates that the enzymes and isozymes from the three classes of human liver alcohol dehydrogenase do not catalyze the oxidation of sorbitol and, further, that a unique sorbitol dehydrogenase is responsible for this reaction. The major form of this human liver sorbitol dehydrogenase has been purified to homogeneity and shown to have a substrate specificity extending to secondary alcohols other than polyhydric alcohols (sugar alcohols).

Experimental Procedures

Materials. NAD^+, NADH (both grade III), the dyes Reactive Green 19 (Green A), Reactive Green 5 (Procion H-4G, Green B), and Coomassie Brilliant Blue G, β-D-

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fructose, and polyol substrates were obtained from Sigma Chemical Co., St. Louis, MO; D-sorbitol (gold label), L-threitol, (2*R*,3*R*)-(−)-2,3-butanediol, (2*S*,3*S*)-(+)-2,3-butanediol, racemic 2,3-butanediol, 4-methylpyrazole, and 1,10-phenanthroline monohydrochloride were from Aldrich Chemical Co., Milwaukee, WI; 1-butanol and 2-butanol were from Fisher Scientific Co., Fair Lawn, NJ; Sepharose CL-4B, SP-Sephadex C-50, and Sephadex G-200 were from Pharmacia Fine Chemicals, Piscataway, NJ; Waters Accell carboxymethyl (CM)[1] cation-exchange medium was from Millipore, Milford, MA; dithiothreitol was from Research Organics, Inc., Cleveland, OH. Dialysis tubing (Spectra/Por 2, Spectrum Medical Industries, Inc., Los Angeles, CA) was pretreated by heating to 70 °C for 1 h with three changes of deionized water, obtained from a Milli-Q Plus reagent water system (Millipore Corp.). The three classes of alcohol dehydrogenases from human liver were purified as described previously (Wagner et al., 1983, 1984; Ditlow et al., 1984).

Dye Chromatography. Triazine dye affinity media were synthesized according to a published procedure (Atkinson et al., 1981). Green A dye was coupled to Sepharose CL-4B as follows: 50 g of the damp gel was suspended in 150 mL of water, and 20 mL of 5 M sodium chloride and 0.75 g of the dye dissolved in 50 mL of water and 5 mL of 5 M sodium hydroxide was added. The suspension was shaken for 72 h at 20 °C and then the uncoupled dye removed by filtration. The particular dyes used in this study were selected with the tandem column technique on the basis of criteria outlined by Scopes (1986, 1987).

Protein Determinations. Protein content was routinely estimated by a Coomassie Blue protein–dye binding assay (Scopes, 1982). Bovine serum albumin ($A^{0.1\%}_{280nm}$ = 0.66) was used as a standard.

Enzyme Assays and Kinetic Analyses. Sorbitol dehydrogenase activity was determined spectrophotometrically by following the absorbance of NADH at 340 nm (ϵ = 6220 M^{-1} cm^{-1}). Measurements were made with a Cary 219 spectrophotometer thermostated to 25 °C. The assay contained 1 mM NAD^+ and 50 mM sorbitol in 0.1 M glycine, pH 10.0. The reaction was usually started by enzyme addition. However, noticeable blank rates in the absence of substrate were observed during the earlier stages of the preparation. In such cases, the reaction was started by addition of substrate. One unit is defined as the amount of enzyme needed to oxidize 1 μmol of substrate per minute under initial velocity conditions in the described assay at 25 °C. Human liver alcohol dehydrogenases were assayed for possible activity with sorbitol by using a Perkin-Elmer MPF-3 fluorescence spectrophotometer at 25 °C. Fluorescence intensity at 460 nm (excitation wavelength 340 nm) was calibrated against NADH solutions, the concentration of which had been determined photometrically. The assay contained 10 mM sorbitol and 2.5 mM NAD^+ in 0.1 M sodium phosphate, pH 7.4. The following alcohol dehydrogenases from human liver were tested: $\alpha\beta_1$, $\alpha\gamma_1$, $\beta_1\beta_1$, $\gamma_1\gamma_1$, π, and χ. Steady-state kinetic parameters were determined from double-reciprocal plots of initial velocity data collected at substrate concentrations ranging from $0.1K_m$ to $10K_m$ when substrate inhibition was not observed.

Analytical Slab Gel Electrophoresis. SDS–PAGE was performed in the discontinuous buffer system described by Laemmli (1970) with a Bio-Rad Model 220 system at 8 °C using phenol red as tracking dye. The gels, 10% in acrylamide, were fixed in acetic acid/methanol/water (7.5:25:67.5 v/v), stained with Coomassie Blue, and dried on filter paper in a Bio-Rad Model 224 slab gel dryer.

pI Determinations. p*I* measurements were done according to the pH-dependent binding analysis reported by Yang and Langer (1985) using SP-Sephadex C-50. The fraction of sorbitol dehydrogenase bound at each pH value was determined by enzymatic assay as described above. The p*I* of the major form of sorbitol dehydrogenase was also determined by analytical isoelectric focusing using a LKB 2117 multiphor electrophoresis system (LKB, Bromma, Sweden) and precast polyacrylamide gels, pH range 3.5–9.5, from LKB.

Metal Analyses. The zinc content of chromatographic fractions was determined by electrothermal atomic absorption spectroscopy using a Perkin-Elmer Model 5000 atomic absorption spectrometer in combination with an HGA 500 graphite furnace programmer and an AS 40 autosampler. Flame atomic absorption spectrophotometry was carried out with a Perkin-Elmer Model 2280 atomic absorption spectrophotometer.

Amino Acid Analyses. Protein samples were hydrolyzed in the vapor phase for 18 h at 110 °C with 6 M hydrochloric acid containing 0.5% (w/v) phenol (Strydom et al., 1985). Amino acid analyses were performed according to the Picotag methodology (Waters Associates; Bidlingmeyer et al., 1984). They are given as an average of four analyses from four different preparations and were not corrected for serine and threonine destruction. Tryptophan analyses were done on a methanesulfonic acid hydrolysate as described previously (Strydom et al., 1986). Cystine/cysteine content was determined as cysteic acid after oxidation with performic acid (Moore, 1963; Strydom et al., 1986).

Purification of Human Liver Sorbitol Dehydrogenase. Human liver was obtained at autopsy (usually within 12 h post-mortem), cut into pieces, and stored at −70 °C. Liver slices totalling 100 g were thawed for 30 min at ambient temperature and homogenized with 100 mL of cold 20 mM sodium phosphate/2 mM magnesium chloride, pH 6.5, in a Waring blender for 10 min and then with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) for 5 min, with the sample on ice. The homogenate was centrifuged for 20 min, 4 °C, at 16000*g*. The supernatant was filtered through glass wool and subsequently passed through a column of Green B–Sepharose CL-4B (26 × 162 mm) equilibrated at ambient temperature with the extraction buffer containing 0.1 mM DTT. All subsequent purification steps were carried out at 4 °C. The pooled active fractions from the Green B affinity column were applied to a column of Green A–Sepharose CL-4B (50 × 105 mm), the column was washed with about 4 column volumes of the same buffer, and the enzymatic activity eluted with a gradient of 0–0.5 M sodium chloride in this buffer. The pooled active fractions were subjected to ammonium sulfate fractionation by addition of solid ammonium sulfate to 45% saturation, maintaining the pH at 7.3 by adding concentrated Tris base. After the last addition, the solution was kept on ice for 30 min. Precipitated protein was removed by centrifugation at 16000*g* for 5 min. The supernatant was then brought to 70% saturation with ammonium sulfate. The pellet was collected by centrifugation, dissolved in 5 mM Hepes/0.1 mM DTT, pH 7.7, and dialyzed against a large excess of the same buffer. After flocculent material was removed by centrifugation, this solution was loaded onto a column (16 × 187 mm) of Accell CM cation-exchange

[1] Abbreviations: CM, carboxymethyl; SP, sulfopropyl; DTT, dithiothreitol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

Table I: Purification of Human Liver Sorbitol Dehydrogenase

step	volume (mL)	protein (mg/mL)	act. (units/mL)	sp act. (units/mg)	overall purification (x-fold)	overall recovery (%)
crude extract[a]	100	28	1.6	0.06		
Green B	70	18	2.1	0.12	2	92
Green A	315	1.2	0.4	0.37	6	79
ammonium sulfate fractionation	20	11	4.2	0.37	6	52[b]
CM-Accell	6.5	0.7	3.6	5.1	85	15
SP-5PW	5	0.3	1.8	6.0[c]	100	6[d]

[a] From 100 g of human liver. [b] 33% of the enzymatic activity was lost upon dialysis after the fractionation. [c] Variation in the specific activity of the enzyme from different livers was observed and ranged from 6 to 13 units/mg. [d] The recovery is based on the pool of the latest eluting peak fractions with sorbitol dehydrogenase activity. Only such pools were used for the physicochemical characterization of the protein.

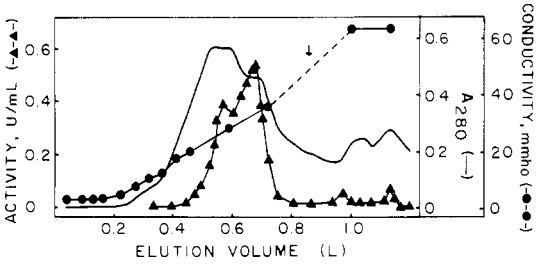


FIGURE 1: Purification of sorbitol dehydrogenase from human liver by triazine dye affinity chromatography (Green A resin). Protein elution was monitored spectrophotometrically at 280 nm (—) with a flow cell of 3-mm light path, sorbitol dehydrogenase activity (▲) was assayed as described under Experimental Procedures, and the shape of the salt gradient (●) was determined by conductivity measurements. The arrow in the figure indicates the onset of isocratic elution with 1 M sodium chloride. Two pools of fractions with sorbitol dehydrogenase activity were formed. The first pool contained fractions of the gradient elution with enzymatic activities greater than 0.05 unit/mL. All the activity from the isocratic elution (after the arrow) was collected in a second pool.

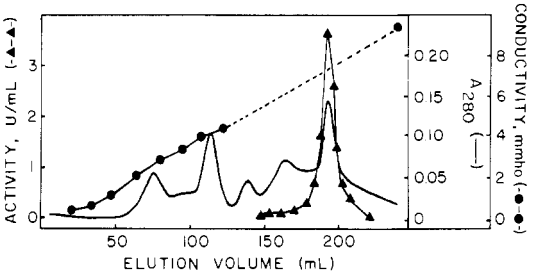


FIGURE 2: Purification of sorbitol dehydrogenase from human liver obtained from the Green A step (see Figure 1) by chromatography on CM-Accell cation-exchange resin. Protein elution (—), enzymatic activity (▲), and the salt gradient (●) were measured as described in the legend of Figure 1. Fractions with sorbitol dehydrogenase activity greater than 0.5 unit/mL were pooled for further purification.

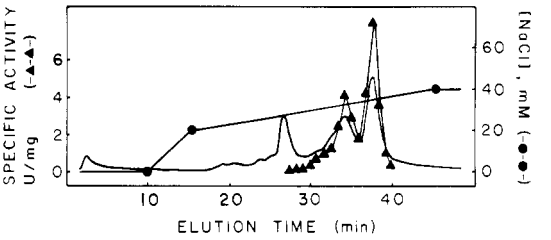


FIGURE 3: Purification of sorbitol dehydrogenase from human liver obtained from the CM-Accell step (Figure 2) by cation-exchange HPLC. Protein elution (—) and enzymatic activity (▲) were determined as described in the legend of Figure 1. The salt gradient (●) is drawn as programmed in the gradient controller; flow-rate, 1 mL/min.

medium. The enzymatic activity was eluted with a gradient of 0–80 mM sodium chloride in the Hepes buffer. Sorbitol dehydrogenase active fractions were concentrated and desalted in Amicon Centricon 30 microconcentrators (Amicon Corp., Danvers, MA). Final purification was achieved by HPLC on a Waters Protein Pak SP-5PW column (7.5 × 75 mm), developed with a 20–40 mM sodium chloride gradient in the Hepes buffer at ambient temperature. Alternatively, the following HPLC gradient system was used: The protein was loaded on the same column in 1 mM sodium phosphate, pH 7.7; the column was washed with this buffer for 5 min followed by a linear gradient from 1 to 10 mM phosphate within 10 min. Sorbitol dehydrogenase was then eluted in a linear gradient from 10 to 25 mM phosphate over 40 min.

Molecular Weight Determination. The oligomeric molecular weight of sorbitol dehydrogenase was determined by gel filtration on a Sephadex G-200 column (16 × 907 mm) that had previously been equilibrated with 50 mM phosphate, pH 6.8, and 0.1 M sodium chloride and calibrated with the following molecular weight markers: blue dextran, ferritin, catalase, aldolase, liver alcohol dehydrogenase, bovine serum albumin, ovalbumin, and chymotrypsinogen.

Results

The major sorbitol-oxidizing enzyme of human liver was purified 100-fold with a yield of 6%. Table I shows the purification protocol. The purification involves two affinity and two cation-exchange chromatographic steps. The first step uses a negative affinity column (Scopes, 1986, 1987) in which the enzymatic activity is not bound. In the next step, sorbitol dehydrogenase activity is bound to the support and eluted with a sodium chloride gradient (Figure 1). At this stage, it was noted that about 10% of the enzymatic activity appears after the main fraction upon isocratic elution with buffer containing 1 M sodium chloride (Figure 1). The elution profile shown in Figure 1 indicates the existence of multiple forms all of which exhibit sorbitol dehydrogenase activity. To test for the existence of different enzymes, a p*I* determination (Yang & Langer, 1985) was performed on the individually pooled active fractions from the gradient and isocratic elutions. The enzymatic activity of the first pool (corresponding to fractions from the gradient elution with an enzymatic activity greater than 0.05 unit/mL) titrated with p*I* values of 8.8 and 7.4 while the second pool (corresponding to the enzymatic activity from the isocratic elution; see Figure 1) yielded values of 7.6 and 6.4, indicating at least three different forms with p*I* values of 6.4, 7.5, and 8.8. The first pool was subjected to ammonium sulfate fractionation between 45% and 70% saturation and purified further by conventional and high-performance cation-exchange chromatography (Figures 2 and 3). After the final HPLC step, the main fraction with sorbitol dehydrogenase activity shows only one band on SDS–PAGE corresponding to a subunit molecular weight of 32000 (Figure 4A). Isoelectric focusing of this form revealed a single band with a p*I* of 9.1. From the elution volume of sorbitol dehydrogenase on a Sephadex G-200 column calibrated with

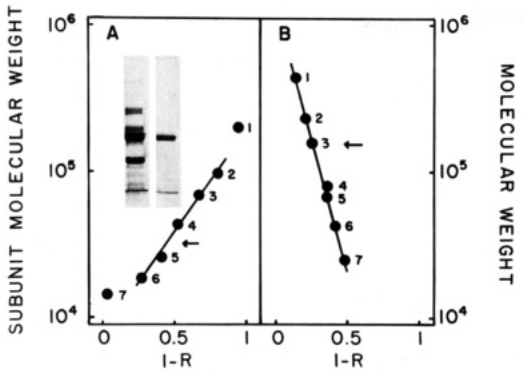


FIGURE 4: (A) Subunit molecular weight determination of sorbitol dehydrogenase by SDS–PAGE. R is defined as the migration distance of the individual protein divided by the migration distance of the front marker. The marker proteins are (1) myosin, (2) phosphorylase b, (3) bovine serum albumin, (4) ovalbumin, (5) chymotrypsinogen A, (6) lactoglobulin, and (7) lysozyme. The arrow indicates the subunit molecular weight of sorbitol dehydrogenase (32000). The inset shows an SDS–PAGE gel with (left lane) the molecular weight markers bovine serum albumin (top), ovalbumin (middle), and bovine erythrocyte carbonic anhydrase (bottom) and (right lane) purified human liver sorbitol dehydrogenase. (B) Molecular weight determination of sorbitol dehydrogenase by gel filtration on Sephadex G-200. R is defined as the elution volume of the individual protein divided by the void volume. The marker proteins are (1) ferritin, (2) catalase, (3) aldolase, (4) human liver alcohol dehydrogenase ($\alpha\beta_1$), (5) bovine serum albumin, (6) ovalbumin, and (7) chymotrypsinogen A. The arrow indicates the molecular weight of sorbitol dehydrogenase (155000).

Table II: Amino Acid Compositions of Human and Sheep Liver Sorbitol Dehydrogenase[a]

amino acid	human liver[b]	sheep liver[c]
Asx	24.6 (25)	27
Glx	27.8 (28)	30
Ser	19.9 (20)	22
Gly	37.8 (38)	34
His	9.1 (9)	10
Arg	15.7 (16)	13
Thr	16.4 (16)	17
Ala	32.4 (32)	29
Pro	25.7 (26)	24
Tyr	7.5 (7)	7
Val	28.4 (28)	36
Met	7.1 (7)	7
Ile	18.9 (19)	18
Leu	35.7 (36)	34
Phe	11.5 (11)	11
Lys	24.6 (25)	23
Cys[d]	8.6 (9)	10
Trp[e]	2.5 (2)	2

[a] Based on a total of 343 amino acid residues, excluding cysteine and tryptophan. [b] Mean of four analyses. [c] From Jeffery et al. (1984b). [d] Determined as cysteic acid. [e] Mean of two analyses; the optical absorption at 280 nm of the enzyme is in agreement with two tryptophan residues per subunit.

molecular weight markers, it is concluded that sorbitol dehydrogenase exhibits a molecular weight of 155000 (Figure 4B). At present, the basis for the resolution of sorbitol dehydrogenase activity into different peaks during the last chromatographic step (Figure 3) is not clear. The fractions with sorbitol dehydrogenase activity which elute earlier were indistinguishable from the main peak of activity, either by SDS–PAGE, by relative rates of oxidation for sorbitol, xylitol, and ribitol, or by amino acid composition. They were also indistinguishable by isoelectric focusing, exhibiting pI values around 9. For the characterization of the enzyme, only fractions from the main peak were pooled.

The amino acid composition of the enzyme is given in Table II together with that reported for the sheep liver enzyme. The human differs most significantly from the sheep enzyme by virtue of the greater number of arginine residues as well as fewer Glx and Asx residues. Although eight less valine residues are found in the human enzyme, this is counterbalanced by an increase in leucine, isoleucine, and alanine residues.

Table III: Steady-State Kinetic Parameters for Human Liver Sorbitol Dehydrogenase[a]

substrate	pH	k_{cat} (s^{-1})	K_m (M)
sorbitol	7.1	1.7	6.2×10^{-4}
	10.0	5.2	6.7×10^{-4}
D-fructose	7.1	45	0.14
xylitol	10.0	5.9	2.2×10^{-4}
L-threitol	10.0	5.3	2.7×10^{-3}
(2R,3R)-2,3-butanediol	10.0	4.2	9.5×10^{-3}

[a] 0.1 M glycine, pH 10.0, or 0.1 M phosphate, pH 7.1, with 1 mM NAD^+ for substrate oxidation or 250 μM NADH for substrate reduction.

Table IV: Substrate Specificity of Human Liver Sorbitol Dehydrogenase

substrate	rel rate (sorbitol = 100%)[a]
glycerol	6
erythritol	4
L-threitol	110
ribitol	85
xylitol	103
L-arabinitol	29
D-mannitol	8
galactitol	11

[a] Assays were carried out with 50 mM substrate and 1 mM NAD^+ in 0.1 M glycine, pH 10.0.

Table V: Enantiomeric Ratios (E) for Epimeric Pairs of Substrates for Human Liver Sorbitol Dehydrogenase

substrate pair	epimer at carbon atom	E[a]
sorbitol/D-mannitol	2	800
xylitol/ribitol	3	20
sorbitol/galactitol	4	500

[a] E is defined as the quotient between k_{cat}/K_m values for each substrate. For D-mannitol and galactitol, it was not possible to obtain k_{cat}/K_m from a full kinetic analysis. Instead, k_{cat}/K_m was determined at low substrate concentrations where $v/E = k_{cat}/K_m[S]$ and where k_{cat}/K_m was established to be independent of substrate concentration. 0.1 M glycine, pH 10.0, and 1 mM NAD^+ were used in these studies.

Steady-state kinetic parameters for substrates of human liver sorbitol dehydrogenase are given in Table III. Interestingly, L-threitol and 2,3-butanediol are substrates for this enzyme. With a racemic mixture of 2,3-butanediol, $k_{cat} = 1.8\ s^{-1}$ and $K_m = 1.25 \times 10^{-2}$ M were determined at pH 10.0. When the two enantiomers of 2,3-butanediol were tested for activity, (2R,3R)-2,3-butanediol turned out to be a substrate (see Table III) while (2S,3S)-2,3-butanediol (up to 40 mM) is not a substrate. The enzyme did not turn over 2-butanol (up to 55 mM). With sorbitol as a substrate at pH 10.0, only 5% of the activity was noted when $NADP^+$ instead of NAD^+ was used as the cofactor. Since the turnover number with sorbitol at pH 10.0 is 3-fold higher than at pH 7.1, the former pH was chosen for routine assays of the enzyme. Table IV lists the relative rates for other polyol substrates. Only L-threitol, ribitol, and xylitol show rates comparable to sorbitol. Enantiomeric ratios for three epimeric substrate pairs have been determined to assess the stereoselectivity of the enzyme toward different carbon atoms of the polyol chain (Table V). At pH 7.0, the enzyme is activated 3-fold upon increasing the ionic strength from 0.02 to 0.5 with sodium chloride either when oxidizing sorbitol or when reducing fructose (data not shown). Sorbitol dehydrogenase oxidizes neither ethanol nor 1-butanol

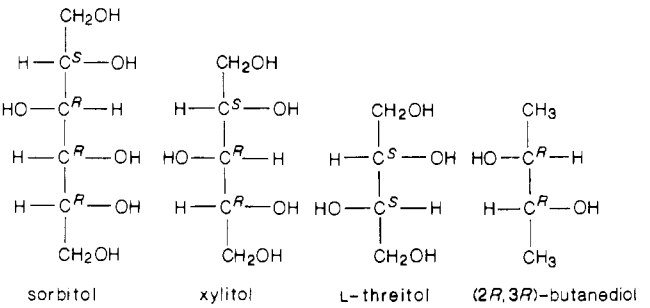
(pH 10.0, 33 mM ethanol or 50 mM 1-butanol/1 mM NAD^+). Conversely, none of the human liver alcohol dehydrogenases, class I (α, β_1, γ_1 subunits), II (π), or III (χ), oxidizes sorbitol (see Experimental Procedures). 1,10-Phenanthroline and also 4-methylpyrazole are competitive inhibitors of sorbitol oxidation with K_i values of 1.5×10^{-4} M and 1.2×10^{-2} M respectively, when assayed in 10 mM phosphate, pH 7.1, and 1 mM NAD^+. At concentrations up to 4 mM, 1,10-phenanthroline acts as a reversible inhibitor.

Zinc analyses of samples from two different preparations yielded values of 0.96 ± 0.26 mol of zinc/mol of subunit by flameless atomic absorption spectroscopy and 0.97 ± 0.10 mol of zinc/mol of subunit by flame atomic absorption spectroscopy. Calculations are based on protein concentrations obtained by the Coomassie Blue protein–dye binding assay as well as amino acid analyses and a subunit molecular weight of 37 700 (see Discussion). On the basis of the protein concentration determined by the dye binding assay on four different preparations, an absorbance coefficient $A^{0.1\%}_{280nm}$ of 0.57 ± 0.13 is calculated.

Discussion

Sorbitol dehydrogenase from human liver closely resembles the enzyme from sheep liver with regard to amino acid composition and a zinc content corresponding to one metal center per subunit. Until recently, there has been a lack of consensus about the oligomeric structure of sorbitol dehydrogenase. Molecular weights reported ranged from 95 000 to 147 000 (Jeffery & Jörnvall, 1988). For the enzyme from sheep liver, the subunit and overall molecular weights have supported a tetrameric model of the enzyme and established an analogy to the tetrameric yeast alcohol dehydrogenase (Jeffery et al., 1981). The present data for sorbitol dehydrogenase from human liver (Figure 4A, B) corroborate such a structure. Human liver alcohol dehydrogenase has been included as a molecular weight marker in the gel filtration experiment (Figure 4B). The elution volume of sorbitol dehydrogenase is not compatible with that of the dimeric alcohol dehydrogenase. The value of 32 000 for the subunit molecular weight of sorbitol dehydrogenase obtained by SDS–PAGE (Figure 4A) is thought to underestimate the subunit size. Data on amino acid analyses (Table II) optimally fit a subunit size of M_r 37 700, and, hence, are in excellent agreement with the overall molecular weight of 155 000 determined by gel filtration.

Human liver sorbitol dehydrogenase has a strict specificity for the secondary alcohol group since enzymatic activity with ethanol or 1-butanol could not be detected. The substrate specificity delineated in the present study, however, differs substantially from that reported for the enzyme from sheep (Eklund et al., 1985) or rat liver (Blakley, 1951; McCorkindale & Edson, 1954). For the former, the following stereochemical requirements for polyol substrates were outlined: (i) presence of a hydroxyl group at C-1; (ii) *S* configuration on C-2; and (iii) *R* configuration on C-4 with the configuration on C-3 and C-5 having very limited influence on activity. The following discussion is based on this numbering of carbon atoms.[2] On the basis of the observation that erythritol is not a substrate for the sheep liver enzyme (Eklund et al., 1985; Smith, 1962), the same was assumed for other tetritols. Therefore, the fact that compounds with a four-carbon chain such as L-threitol



and (2*R*,3*R*)-2,3-butanediol have been found here to serve as substrates for the enzyme from human liver establishes a new class of substrates for sorbitol dehydrogenase. Further, the very low activity of the human liver enzyme toward erythritol and its high activity toward the diastereoisomer L-threitol demonstrate the critical importance of the relative configurations at the asymmetric carbon atoms for this type of substrate. In addition, the activity of the human liver enzyme toward (2*R*,3*R*)-2,3-butanediol and the absence of activity with the corresponding enantiomer constitute a reversal in enantioselectivity with respect to L-threitol and the other polyol substrates with opposite configuration at C-2, and, hence, indicate a different binding mode of this substrate. It was also observed that 1-deoxysorbitol is not a substrate for the sheep liver enzyme. Therefore, a hydroxyl group at C-1 was thought to be essential for polyol substrates (Eklund et al., 1985). Since 2,3-butanediol is a substrate for the human enzyme, a hydroxyl group at C-1 is not required for compounds with a four-carbon chain. Obviously, compounds other than polyhydric alcohols can serve as substrates for this enzyme. In order to evaluate the stereospecificity of the enzyme at carbon atoms C-2, C-3, and C-4 of hexitols and pentitols in a quantitative manner, enantiomeric ratios (*E*) for different substrate pairs were determined (Table V). The high value of *E* for carbon atoms 2 and 4 and the low value of *E* for carbon atom 3 support the required stereochemistry of substrates at these carbon atoms as summarized above for the sheep liver enzyme (Eklund et al., 1985). Since the enzyme has been named L-iditol 5- or 2-oxidoreductase,[3] it is implicit that some polyol substrates can be oxidized at either end of the chain. Therefore, the configuration at C-5 or, more generally, at the penultimate carbon atom turns out to be an important factor. It is apparent from this discussion that the stereoselectivity of sorbitol dehydrogenase will be assessed adequately only in conjunction with careful product analyses, especially if oxidation can occur at both ends of the polyol chain. The K_m for substrates increases with decreasing chain length (see Table III). Since glycerol is a very poor substrate, a chain length of four carbon atoms seems to be a minimum requirement. The high K_m for D-fructose suggests that the free ketose, representing only 0.8% of the substrate at 20 °C (Angyal, 1984), is the catalytically competent structure. Presumably, the furanose and pyranose ring structures of D-fructose are not attacked.

On the basis of seven successive preparations, the total sorbitol dehydrogenase activity in human liver is estimated to be about 1000 units. This relatively high amount of the enzyme raises questions of its metabolic role. Sorbitol dehydrogenase together with aldose reductase (alditol:$NADP^+$ 1-oxidoreductase, EC 1.1.1.21) constitutes the initial steps in the polyol pathway, a bypass to glycolysis (Jeffery & Jörnvall, 1983):

[2] One of the reviewers pointed out that the numbering of the carbon atoms in erythritol is ambiguous. In such a case, rules for stereospecific numbering (Hirschmann & Hanson, 1971) should be applied.

[3] The 1984 edition of Enzyme Nomenclature lists the enzyme as L-iditol:NAD^+ 2-oxidoreductase because L-iditol possesses rotational symmetry.

$$\text{D-glucose} \underset{\text{aldose reductase}}{\overset{\text{NADPH/NADP}}{\rightleftharpoons}} \text{sorbitol} \underset{\text{sorbitol dehydrogenase}}{\overset{\text{NAD/NADH}}{\rightleftharpoons}} \text{D-fructose}$$

Earlier reports suggested that the second enzyme of the polyol pathway, i.e., aldose reductase, does not exist in the liver (Hers, 1960). However, immunohistochemical methods have recently localized this enzyme to hepatic tissue (Wirth & Wermuth, 1985), demonstrating that the polyol pathway could operate in the liver. Functions of this pathway in transhydrogenation, i.e., transfer of reducing equivalents between NAD^+ and $NADP^+$, or regulation of osmotic pressure have been discussed (Jeffery & Jörnvall, 1988; Clements, 1986).

While only sorbitol and xylitol are considered endogenous polyols, other acyclic polyols occur naturally in foods and are used as sweeteners, as food additives, or in pharmaceutical or cosmetic preparations (Emodi, 1982). The metabolism of these polyols is not well understood, and presently it is not clear if these compounds require other, additional polyol dehydrogenases for their oxidation. Therefore, we will restrict our discussion here to sorbitol and xylitol.

Sorbitol was recently identified as an osmolyte; it was noted that in cultured renal inner medullary cells up to 0.24 M sorbitol accumulates intracellularly to balance the osmotic pressure of 0.3 M sodium chloride in the medium. Aldose reductase induction was thought to be a response to extracellular sodium chloride (Bagnasco et al., 1987). The participation of sorbitol dehydrogenase, known to occur in kidney, was not considered in the process of controlling the sorbitol concentration of cells in regard to chloride. In this context, we note the increase of sorbitol dehydrogenase activity with increasing chloride concentration (see Results) which could be of physiological importance.

Xylitol is known to be oxidized to L-xylulose by NADP-dependent L-xylulose reductase (EC 1.1.1.10) or to D-xylulose by NAD-dependent xylitol dehydrogenase (EC 1.1.1.9). The latter has been reported to occur in both a mitochondrial and a cytoplasmic form (Arsenius & Touster, 1969). Furthermore, the mitochondrial form has been thought to be a zinc enzyme and shown to oxidize L-threitol. In all other ways, the enzyme was found to be homospecific with the corresponding cytoplasmic enzyme (Hollmann, 1959). The kinetic data for xylitol and liver sorbitol dehydrogenase presented here support the idea that this enzyme is identical with the cytoplasmic xylitol dehydrogenase. Therefore, for the human liver sorbitol dehydrogenase, the designation polyol dehydrogenase may be more appropriate.

In a hypothesis termed "A Stereochemical Imperative in Dehydrogenases", the equilibrium constant of a dehydrogenase-catalyzed reaction was correlated with the stereochemistry of hydrogen transfer from NADH, separating dehydrogenases with $pK_{eq} > 11$, grouped as *pro-R* specific, from those that are *pro-S* specific with $pK_{eq} < 11$ (Benner et al., 1985). The equilibrium constant reported for the substrate pair sorbitol/D-fructose is $pK_{eq} \sim 8.6$ (Blakley, 1951). According to the above-mentioned stereochemical imperative, this equilibrium constant would assign sorbitol dehydrogenase *pro-S* specificity. However, sheep liver sorbitol dehydrogenase has been classified as *pro-R* specific (Englard et al., 1970). If the correlation of Benner and co-workers is assumed to prevail, it would have to be concluded that sorbitol/D-fructose is not the physiologically important substrate pair for this enzyme. The relatively low values of the specificity parameter, k_{cat}/K_m, for both sorbitol (2.7×10^3 M^{-1} s^{-1}) and D-fructose (3.2×10^2 M^{-1} s^{-1}) measured in the present study at pH 7.1 would seem to corroborate such a conclusion. For comparison, k_{cat}/K_m for benzyl alcohol and the $\gamma_1\gamma_1$ isozyme of human liver alcohol dehydrogenase, which has been determined as 3×10^5 M^{-1} s^{-1} (Wagner et al., 1983), provides an example for high specificity in alcohol dehydrogenases.

Evidence for polymorphic forms of sorbitol dehydrogenase from various species, largely resting on the observation of electrophoretic variants, has been summarized (Jeffery & Jörnvall, 1988). Two forms with p*I* values of 6.2 and 9.5 have been reported previously for human liver (Nealon & Rej, 1983). Our present data, however, indicate three forms of human liver sorbitol dehydrogenase with p*I* values of 6.4, 7.5, and 8.8 (see Figure 1 and Results). The existence of three forms in one species is consistent with the classification of sorbitol dehydrogenases from different species into three groups (Porter & McGuinness, 1987). Such a pattern of enzymes is reminiscent of the three classes of alcohol dehydrogenases (Vallee & Bazzone, 1983) whose characteristic p*I* values are also in the same range. Further characterization of the different forms of human liver sorbitol dehydrogenase is necessary to establish whether true isozymes exist.[4]

Since sorbitol dehydrogenase shares the cytoplasmic coenzyme pool with alcohol dehydrogenases, substrates for one enzyme may adversely affect the action of the other enzyme. Thus, it has been known for about 50 years that the oral administration of D-fructose accelerates ethanol turnover (Carpenter & Lee, 1937). Several explanations have been given for this so-called "fructose-effect". It has been suggested (i) that a stimulation of cytoplasmic NADH oxidation is achieved by glyceraldehyde derived from D-fructose acting as a substrate upon alcohol dehydrogenase when NADH is still bound to the enzyme (Holzer & Schneider, 1955), (ii) that an increased mitochondrial oxidation of NADH takes place (Krebs & Stubbs, 1975; Scholz & Nohl, 1976), or (iii) that shuttle systems are affected (Grunnet et al., 1973; Berry & Kun, 1978). The amount of sorbitol dehydrogenase activity in liver, however, lends support to the hypothesis (Berry & Kun, 1978) that the sorbitol dehydrogenase catalyzed reduction of fructose provides NAD^+ for ethanol oxidation. The relatively high K_m value for fructose (see Table III) is in agreement with the observation that a large amount of fructose is needed to enhance ethanol metabolism. With the availability of human liver sorbitol dehydrogenase, it is now possible to test whether this enzyme is responsible for the "fructose-effect".

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Registry No. Zn, 7440-66-6; (±)-Me(CHOH)$_2$Me, 6982-25-8; (2*R*,3*R*)-(−)-Me(CHOH)$_2$Me, 24347-58-8; (2*S*,3*S*)-(+)-Me(CHOH)$_2$Me, 19132-06-0; sorbitol, 50-70-4; D-fructose, 57-48-7; xylitol, 87-99-0; L-threitol, 2319-57-5; 1,10-phenanthroline, 66-71-7; 4-methylpyrazole, 7554-65-6; sorbitol dehydrogenase, 9028-21-1.

REFERENCES

Angyal, S. J. (1984) *Adv. Carbohydr. Chem. Biochem. 42*, 15–68.

[4] We note that the pH-dependent binding analysis (Yang & Langer, 1985) for the determination of isoelectric points of proteins is prone to give displaced p*I* values as a result of ligand binding to the protein. For instance, we have applied the method to the determination of the p*I* value of the horse liver alcohol dehydrogenase EE isozyme and found p*I* = 6.9 in contrast to the true p*I* = 8.7 reported (Lutstorf et al., 1970). Therefore, it is possible that the p*I* = 7.5 form of sorbitol dehydrogenase represents an enzyme/ligand complex.

Arsenius, C., & Touster, O. (1969) *J. Biol. Chem. 244*, 3895–3899.
Atkinson, T., Hammond, P. M., Hartwell, R. D., Hughes, P., Scawen, M. D., Sherwood, R. F., Small, D. A. P., Bruton, C. J., Harvey, M. J., & Lowe, C. R. (1981) *Biochem. Soc. Trans. 9*, 290–293.
Bagnasco, S. M., Uchida, S., Balaban, R. S., Kador, P. F., & Burg, M. B. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1718–1720.
Barretto, O. C. O., Nomoyama, K., Federsoni, P., Fedullo, J. D. L., & Diniz, L. B. S. M. (1985) *Comp. Biochem. Physiol., B: Comp. Biochem. 82B*, 317–319.
Benner, S. A., Nambiar, K. P., & Chambers, G. K. (1985) *J. Am. Chem. Soc. 107*, 5513–5517.
Berry, M. N., & Kun, E. (1978) *Eur. J. Biochem. 89*, 237–241.
Bidlingmeyer, B. A., Cohen, S. A., & Tarvin, T. L. (1984) *J. Chromatogr. 336*, 93–104.
Blakley, R. K. (1951) *Biochem. J. 49*, 257–271.
Carpenter, T. M., & Lee, R. C. (1937) *J. Pharmacol. Exp. Ther. 60*, 286–295.
Clements, R. S., Jr. (1986) *Drugs 32 (Suppl. 2)*, 3–5.
Ditlow, C. C., Holmquist, B., Morelock, M., & Vallee, B. L. (1984) *Biochemistry 23*, 6363–6368.
Eklund, H., Horjales, E., Jörnvall, H., Brändén, C.-I., & Jeffery, J. (1985) *Biochemistry 24*, 8005–8012.
Emodi, A. (1982) in *Food Carbohydrates* (Lineback, D. R., & Inglett, G. E., Eds.) pp 49–61, AVI, Westport, CT.
Englard, S., Kaysen, G., & Avigad, G. (1970) *J. Biol. Chem. 245*, 1311–1318.
Gabbay, K. H. (1973) *N. Engl. J. Med. 288*, 831–836.
Gerlach, U., & Hiby, W. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.) pp 569–573, Verlag Chemie, Weinheim, FRG.
Greene, D. A., Lattimer, S. A., & Sima, A. A. F. (1987) *N. Engl. J. Med. 316*, 599–606.
Grunnet, N., Quistorff, B., & Thieden, H. I. D. (1973) *Eur. J. Biochem. 40*, 275–282.
Holzer, H., & Schneider, S. (1955) *Klin. Wochenschr. 33*, 1006–1009.
Hers, H. G. (1960) *Biochim. Biophys. Acta 37*, 127–138.
Hirschmann, H., & Hanson, K. R. (1971) *Eur. J. Biochem. 22*, 301–309.
Hollmann, S. (1959) *Hoppe-Seyler's Z. Physiol. Chem. 317*, 193–215.
Höög, J.-O., von Bahr-Lindström, H., Hedén, L.-O., Holmquist, B., Larsson, K., Hempel, J., Vallee, B. L., & Jörnvall, H. (1987) *Biochemistry 26*, 1926–1932.
Jedziniak, J. A., Chylack, L. T., Jr., Cheng, H.-M., Gillis, M. K., Kalustian, A. A., & Tung, W. H. (1981) *Invest. Ophthalmol. Visual Sci. 20*, 314–326.
Jeffery, J., & Jörnvall, H. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 901–905.
Jeffery, J., & Jörnvall, H. (1988) *Adv. Enzymol. Relat. Areas Mol. Biol.* (in press).
Jeffery, J., Cummins, L., Carlquist, M., & Jörnvall, H. (1981) *Eur. J. Biochem. 120*, 229–234.
Jeffery, J., Chesters, J., Mills, C., Sadler, P. J., & Jörnvall, H. (1984a) *EMBO J. 3*, 357–360.
Jeffery, J., Cederlund, E., & Jörnvall, H. (1984b) *Eur. J. Biochem. 140*, 7–16.
Jörnvall, H., Persson, M., & Jeffery, J. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4226–4230.
Jörnvall, H., Höög, J.-O., von Bahr-Lindström, H., & Vallee, B. L. (1987a) *Proc. Natl. Acad. Sci. U.S.A. 84*, 2580–2584.
Jörnvall, H., Hempel, J., & Vallee, B. L. (1987b) *Enzyme 37*, 5–18.
Kaiser, R., Holmquist, B., Hempel, J., Vallee, B. L., & Jörnvall, H. (1988) *Biochemistry 27*, 1132–1140.
Krebs, H. A., & Stubbs, M. (1975) *Adv. Exp. Med. Biol. 59*, 149–161.
Laemmli, U. K. (1970) *Nature (London) 277*, 680–685.
Lutstorf, U. M., Schurch, P. M., & von Wartburg, J. P. (1970) *Eur. J. Biochem. 17*, 497–508.
McCorkindale, J., & Edson, N. L. (1954) *Biochem. J. 57*, 518–523.
Moore, S. (1963) *J. Biol. Chem. 238*, 235–237.
Nealon, D. A., & Rej, R. (1983) in *Selected Topics in Clinical Enzymology* (Werner, M., & Goldberg, D. M., Eds.) Vol. 2, pp 535–544, de Gruyter, New York.
O'Brien, M. M., Schofield, P. J., & Edwards, M. R. (1983) *Biochem. J. 211*, 81–90.
Porter, G. F., & McGuinness, E. T. (1987) *Prep. Biochem. 17*, 143–155.
Scholz, R., & Nohl, H. (1976) *Eur. J. Biochem. 63*, 449–458.
Scopes, R. K. (1982) *Protein Purification*, p 266, Springer, New York.
Scopes, R. K. (1986) *J. Chromatogr. 376*, 131–140.
Scopes, R. K. (1987) *Anal. Biochem. 165*, 235–246.
Smith, M. G. (1962) *Biochem. J. 83*, 135–144.
Strydom, D. J., Fett, J. W., Lobb, R. R., Alderman, E. M., Bethune, J. L., Riordan, J. F., & Vallee, B. L. (1985) *Biochemistry 24*, 5486–5494.
Strydom, D. J., Harper, J. W., & Lobb, R. R. (1986) *Biochemistry 25*, 945–951.
Vallee, B. L., & Bazzone, T. J. (1983) *Isozymes: Curr. Top. Biol. Med. Res. 8*, 219–244.
Wagner, F. W., Burger, A. R., & Vallee, B. L. (1983) *Biochemistry 22*, 1857–1863.
Wagner, F. W., Parés, X., Holmquist, B., & Vallee, B. L. (1984) *Biochemistry 23*, 2193–2199.
Wirth, H. P., & Wermuth, B. (1985) in *Enzymology of Carbonyl Metabolism* (Flynn, T. G., & Weiner, H., Eds.) pp 231–239, Liss, New York.
Yang, V. C., & Langer, R. (1985) *Anal. Biochem. 147*, 148–155.