Biochem. J. (1983) **211**, 81–90
Printed in Great Britain

Polyol-pathway enzymes of human brain

Partial purification and properties of sorbitol dehydrogenase

Margaret M. O'BRIEN, Philip J. SCHOFIELD and Michael R. EDWARDS
School of Biochemistry, University of New South Wales, P.O. Box 1, Kensington, N.S.W. 2033, Australia

(*Received 12 August 1982/Accepted 14 December 1982*)

Sorbitol dehydrogenase was isolated from human brain and purified 690-fold, giving a final specific activity of 11.1 units/mg of protein. The enzyme preparation was nearly homogeneous, but was unstable at most temperatures. It exhibited a broad pH optimum of 7.5–9.0 in the forward reaction (i.e. sorbitol→fructose), and of 7.0 in the reverse reaction (i.e. fructose→sorbitol). Substrate-specificity studies demonstrated that the enzyme had the capability to oxidize a wide range of polyols and that the enzyme had a higher affinity for substrates in the forward reaction than in the reverse reaction, e.g. K_m for sorbitol was 0.45 mM, and that for fructose was 480 mM. However, the $V_{max.}$ was 10 times greater in the reverse reaction. At high concentrations of fructose (500 mM) the enzyme exhibited substrate inhibition in the reverse reaction. The enzyme mechanism was sequential, as determined by the kinetic patterns arising from varying the substrate concentrations. In addition, both fructose and NADH protected the enzyme against thermal inactivation. These findings, together with product-inhibition data, suggested that the mechanism is random rapid equilibrium with two dead-end complexes.

The polyol pathway is a minor pathway of glucose metabolism that assumes a major role under certain conditions, such as diabetic hyperglycaemia. It consists of two successive dehydrogenases, namely aldose reductase (alditol:$NADP^+$ 1-oxidoreductase, EC1.1.1.21) and sorbitol dehydrogenase (L-iditol:NAD^+ 5-oxidoreductase, EC 1.1.1.14). In human brain, there is a second aldose-reducing enzyme, L-hexonate dehydrogenase (L-gulonate:$NADP^+$ 1-oxidoreductase, EC 1.1.1.19), with the potential to synthesize polyols (O'Brien & Schofield, 1980).

Under normal conditions, there is little flux through the polyol pathway. However, under conditions of diabetic hyperglycaemia, the flux through the pathway increases, as there is an overflow of glucose from the normal metabolic pathways such as glycolysis. This increased flux may lead to an accumulation of the intermediate of the pathway, sorbitol, which is unable to cross cell membranes. A large increase in the intracellular polyol concentration can lead to an osmotic imbalance, causing the cells to imbibe extracellular water and thus swell. The resulting cellular disruption is known, or is suggested, to be responsible for a number of pathological conditions in various tissues, such as cataracts in the lens and neuropathy in peripheral nerves (for review see Winegrad *et al.*, 1972). It has also been suggested that this series of events in the brain could lead to cerebral oedema (Clements *et al.*, 1968; Prockop, 1971). Similar circumstances arise in galactosaemia as a result of the synthesis and intracellular accumulation of the corresponding polyol, galactitol.

The polyol accumulation arises from an imbalance in the flux through the enzymes of the pathway. To try to understand how this imbalance occurs we have attempted to characterize the enzymes concerned from human brain, and have previously reported some properties of the human brain aldose reductase and hexonate dehydrogenase. The present paper describes the partial purification of the human brain sorbitol dehydrogenase. The enzyme has been partially characterized and a mechanism is proposed, thus allowing comparison with properties described for sorbitol dehydrogenase from other sources. These include sheep liver (Smith, 1962), calf lens (Jedziniak *et al.*, 1973), human erythrocytes (Barretto & Beutler, 1975), rat brain (Rehg & Torack, 1977), rat liver (Leissing & McGuinness, 1978) and human lens (Jedziniak, 1980; Jedziniak *et al.*, 1981).

Materials

Human brains were obtained from the Department of Pathology, Prince of Wales Hospital,

Randwick, N.S.W., Australia. The tissue was washed in 0.9% NaCl and stored at −20°C until required.

L-Arabitol, Bromophenol Blue, cysteine, galactitol, D-mannitol, *p*-chloromercuribenzoate, phenazine methosulphate, protamine sulphate, quercetin, ribitol, sorbitol, L-sorbose, Tetrazolium Violet and xylitol were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. NAD^+, NADH, $NADP^+$ and NADPH were purchased from Boehringer Mannheim, Sydney, N.S.W., Australia. Bovine serum albumin, D-fructose, 2-mercaptoethanol and triethanolamine were obtained from Calbiochem, Sydney, N.S.W., Australia, and 1,10-phenanthroline was from Hopkin and Williams, Chadwell Heath, Essex, U.K.

DEAE-cellulose (DE-52) and CM-cellulose (CM-52) were obtained from Whatman, Maidstone, Kent, U.K., and Sephadex G-25 was from Pharmacia Fine Chemicals, Uppsala, Sweden. Matrex Red A gel was purchased from Amicon, Membrane Filtration Industries, Adelaide, S. Australia, Australia.

Methods

Enzyme assay

Activity of sorbitol dehydrogenase was determined by measurement of the change in absorbance at 340nm at 30°C as NADH was oxidized or NAD^+ reduced. The physiological direction, the conversion of sorbitol into fructose, was designated the 'forward reaction', and the conversion of fructose into sorbitol was termed the 'reverse reaction'. During the purification procedure the enzyme was assayed by using the reverse reaction, and the assay mixture contained 150mM-triethanolamine/HCl buffer, pH 7.4, 200mM-fructose, 100μM-NADH and enzyme in a total volume of 3.0ml.

When sorbitol dehydrogenase activity was measured in the forward direction, the assay mixture contained 30mM-sodium pyrophosphate buffer, pH 9.0, 6mM-sorbitol, 200μM-NAD^+ and enzyme in a total volume of 3.0ml. The reaction was initiated by addition of enzyme, with the appropriate controls being performed. An enzyme unit was defined as the amount of enzyme oxidizing 1μmol of NADH/min at 30°C in the reverse reaction under the assay conditions. Linearity was observed for at least 5min after initiation of the reaction, and velocity was proportional to protein concentration.

Protein determination

Protein concentrations were determined by the micro biuret method of Itzhaki & Gill (1964). Bovine serum albumin was used as the protein standard. Column eluents were continuously monitored for protein by measurement of the absorbance at 280nm.

Polyacrylamide-gel electrophoresis

Polyacrylamide gels (7%) were prepared as described by Davis (1964). The marker dye was 0.01% Bromophenol Blue, and the samples were applied in 10% (w/v) glycerol.

The method of Chrambach *et al.* (1967) was employed to observe protein migration. Enzyme activity was detected by two methods. The first method involved staining with Tetrazolium Violet dye. Gels were immersed in 30mM-sodium pyrophosphate buffer, pH 9.0, for 10min and then incubated in the reaction mixture for 30min in the dark at 37°C. The reaction mixture contained 30mM-sodium pyrophosphate buffer, pH 9.0, 30mM-sorbitol, 1mM-NAD^+, 0.02mg of phenazine methosulphate/ml and 0.35mg of Tetrazolium Violet/ml. Enzyme activity was indicated by the development of a fluorescent pink band. The second method involved slicing the gel into 1mm sections and incubating each slice in 0.2ml of assay mixture at 25°C. This assay mixture contained 150mM-triethanolamine/HCl buffer, pH 7.4, 0.2M-fructose and 0.2mM-NADH. After 30min, 1ml of water was added to each slice mixture and the absorbance was measured at 340nm. The portion of gel corresponding to a decrease in absorbance (i.e. NADH oxidized) contained the enzyme.

Purification procedure

All steps were performed at 0–4°C.

1. *Preparation of crude extract.* Approx. 500g of human brain (fresh or frozen) was homogenized in a Waring blender in 2ml of 20mM-sodium phosphate/5mM-2-mercaptoethanol buffer, pH 7.4, per g of tissue. The homogenate was centrifuged at 18000*g* for 75min, and the precipitate of solid brain matter was discarded.

2. *Protamine sulphate fractionation.* An aqueous solution of 1% (w/v) protamine sulphate was added to the crude extract (1ml/2ml of extract) over a period of 15min. The pH was monitored and maintained at pH 7.0 with 0.1M-NaOH. After an additional 5min of stirring, the precipitate was removed by centrifugation at 18000*g* for 30min and discarded.

3. *Ammonium sulphate precipitation.* Solid $(NH_4)_2SO_4$ (242g/l) was slowly added with stirring to the supernatant from the previous step until 40% saturation was obtained. After a further 15min stirring, the suspension was centrifuged at 18000*g* for 30min, and the precipitate was discarded. The supernatant was adjusted slowly to 60% saturation with the appropriate mass of solid $(NH_4)_2SO_4$ (131g/l), stirred for an additional 30min and re-centrifuged. The resulting precipitate was dissolved in approx. 20ml of 10mM-sodium phosphate/1mM-2-mercaptoethanol buffer, pH 7.2, and

dialysed overnight against 4 litres of the same buffer. All buffers used in further stages of purification contained 1 mM-2-mercaptoethanol.

4. *DEAE-cellulose chromatography.* The dialysed solution was applied to a DEAE-cellulose column (3 cm × 40 cm) that had been previously equilibrated with 10 mM-sodium phosphate buffer, pH 7.2. This same buffer was then used to elute the enzyme, which did not bind to the column. The flow rate was 120 ml/h, and 10 ml fractions were collected. The enzyme activity was concentrated by a 0–75%-saturation-$(NH_4)_2SO_4$ (560 g/l) precipitation. The precipitate was redissolved in approx. 4 ml of 20 mM-sodium phosphate/1 mM-2-mercaptoethanol buffer, pH 6.2, and dialysed overnight against 2 litres of the same buffer.

5. *CM-cellulose chromatography.* The dialysed enzyme solution was then applied to a CM-cellulose column (1.5 cm × 25 cm) that had been previously equilibrated with 20 mM-sodium phosphate/1 mM-2-mercaptoethanol buffer, pH 6.2. The column was then washed with this same buffer, and a 300 ml linear gradient of 20–100 mM-sodium phosphate buffer, pH 6.2, was used to elute the enzyme. The flow rate was 80 ml/h, and 5 ml fractions were collected. Active fractions were again concentrated by 0–80%-saturation-$(NH_4)_2SO_4$ (560 g/l) precipitation, the precipitate being redissolved in approx. 2 ml of 10 mM-sodium phosphate buffer, pH 7.5. This solution was then desalted on a Sephadex G-25 column (1.5 cm × 15 cm) previously equilibrated with 10 mM-sodium phosphate buffer, pH 7.5 (flow rate 160 ml/h; fraction size 3 ml). Active fractions were combined and used immediately in the next purification step.

6. *Affinity chromatography.* The desalted enzyme solution was applied to a 5 ml Matrex Red A-gel column previously equilibrated to 10 mM-sodium phosphate buffer, pH 7.5. The column was then washed with 30 ml of this buffer. An 80 ml linear gradient of 0–1.5 M-KCl in 10 mM-sodium phosphate buffer, pH 7.5, was used to elute the enzyme. The flow rate was 7 ml/h, and 1.2 ml fractions were collected. Active fractions were combined and concentrated by using an Amicon micro-ultrafiltration unit under N_2 (400 kPa) with a 5000-M_r cut-off membrane. The enzyme was then stored at −70°C in 2 M-$(NH_4)_2SO_4$.

Kinetic experiments

The enzyme used in the determination of substrate specificity and kinetic mechanism was activity eluted from the CM-cellulose column after concentration, with a specific activity of 1.2–2.0 units/mg of protein.

All kinetic data were analysed by the appropriate computer program listed by Cleland (1979) to obtain values for constants in the rate equation, and inhibitor constants.

Results and discussion

Enzyme purification

Sorbitol dehydrogenase is present in human brain at an activity of approx. 0.2 unit/g wet wt. of fresh brain. However, when the brain is stored at −20°C, the activity of the enzyme slowly decreases, losing up to 50% of the initial activity over a period of 6 months. For this reason, brain was normally used within 2 months of freezing. A typical purification of human brain sorbitol dehydrogenase is presented in Table 1. The enzyme was purified 690-fold with a 5.5% yield, giving a final specific activity of 11.1 units/mg of protein. The enzyme did not bind to DEAE-cellulose at pH 7.2. The reason that a large DEAE-cellulose column was used in the purification procedure was primarily because it was simultaneously used for the isolation of the other

Table 1. *Purification of sorbitol dehydrogenase from human brain*
For full experimental details see the text.

Purification step	Volume (ml)	Concn. of protein (mg/ml)	Total protein (mg)	Specific activity (units/mg of protein)	Total activity (units)	Yield (%)	Purification (fold)
1. Crude extract	735	8.6	6320	0.0161	102	100	1
2. Protamine sulphate precipitation	770	7.8	6010	0.0169	102	100	1
3. 40–60%-satn.-$(NH_4)_2SO_4$ fraction	34.0	55.5	1890	0.0452	85.4	84	2.8
4. DEAE-cellulose column eluate	55.5	5.45	302	0.216	65.3	64	13.4
5. CM-cellulose column eluate	42.5	0.38	16.2	1.71	27.6	27	106
6. Matrex Red A-gel column eluate	7.9	0.08	0.63	10.8	6.8	7	671
7. After ultrafiltration	1.0	0.51	0.51	11.1	5.7	5.5	690

polyol-pathway enzymes, which are eluted after the wash containing the sorbitol dehydrogenase. The purification of these other enzymes, namely aldose reductase and hexonate dehydrogenase, has been previously described (O'Brien & Schofield, 1980).

Human brain sorbitol dehydrogenase bound to CM-cellulose at pH 6.2. A typical elution profile is shown in Fig. 1. The enzyme also bound to Matrex Red A gel at pH 7.5 and could be eluted with a 0–1.5 M-KCl gradient. The final sorbitol dehydrogenase preparation gave two protein bands on polyacrylamide-gel electrophoresis. The major band nearer the origin was identified as sorbitol dehydrogenase both by Tetrazolium Violet staining and by measurement of activity. The only other brain sorbitol dehydrogenase previously purified is that from rat (Rehg & Torack, 1977), but in that case the final specific activity was only 2.0 units/mg of protein.

Human brain sorbitol dehydrogenase was unstable, especially in dilute solution (less than approx. 1 mg of protein/ml). For this reason the enzyme purification needed to be completed as quickly as possible (within 72 h). The final preparation lost 50% of activity in 2 days at 4°C, and 50% in 20 days at −20°C. However, no activity was lost at −70°C for at least 6 months. This is not the first recorded case of sorbitol dehydrogenase exhibiting lability; similar lack of stability has been described for the enzyme from a number of different sources, e.g. sheep liver (Smith, 1962) and rat brain (Rehg & Torack, 1977). Bovine serum albumin (0.2%) had no stabilizing effect on the human brain enzyme at 4°C, in contrast with the rat brain sorbitol dehydrogenase, where bovine serum albumin preserved enzyme activity for 1 week at this temperature (Rehg & Torack, 1977).

pH optimum

The human brain enzyme exhibited a broad pH optimum between 7.5 and 9.0 in the forward direction, i.e. sorbitol → fructose. In the reverse direction, i.e. fructose → sorbitol, the pH optimum was 7.0 (Fig. 2). These values are similar to those found for the enzyme from human lens (Jedziniak *et al.*, 1981) and from rat liver (Leissing & McGuinness, 1978). The pH optimum values quoted for the enzyme from sheep liver (Smith, 1962), calf lens (Jedziniak *et al.*, 1973) and rat brain (Rehg & Torack, 1977) are all slightly higher, being in the range 9.0–10.0 in the forward direction, and in the range 7.0–8.0 in the reverse direction.

Below pH 6, the enzyme was inactivated within 10 min at 4°C, with complete inactivation occurring almost instantaneously at pH 4. However, in the pH range 6–10 the effects of pH were completely reversible. Furthermore, increasing the ionic strength by the addition of 0.5 M-NaCl did not alter the pH–activity profile, which suggests that the observed pH effects are not significantly affected by changes in the buffer ionic strength.

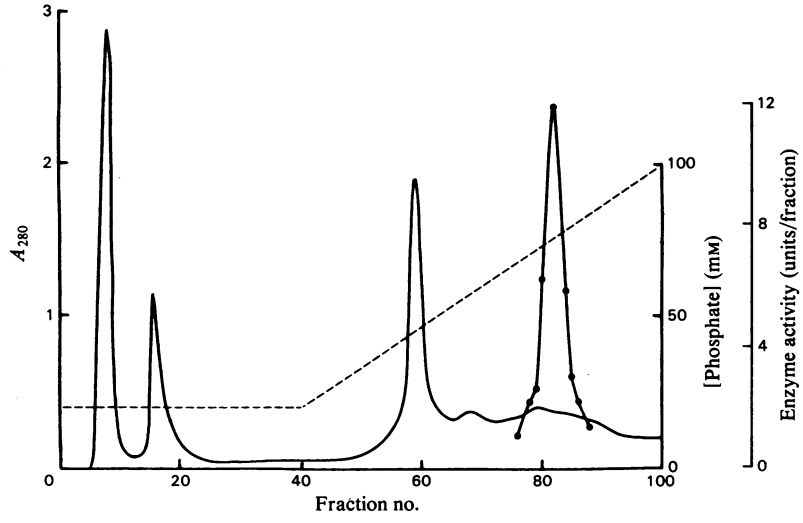


Fig. 1. *Purification of human brain sorbitol dehydrogenase on a CM-cellulose column (1.5 cm × 25 cm) at pH 6.2*
The flow rate was 80 ml/h, and 5 ml fractions were collected. Protein was continuously monitored by measuring the absorbance of the eluate at 280 nm. Sorbitol dehydrogenase assay mixtures (●) contained 150 mM-triethanolamine/HCl buffer, pH 7.4, 200 mM-fructose, 100 μM-NADH and 25 μl of fraction in a total volume of 3.0 ml. ●, Sorbitol dehydrogenase activity; ----, sodium phosphate buffer concentration; ——, A_{280}.

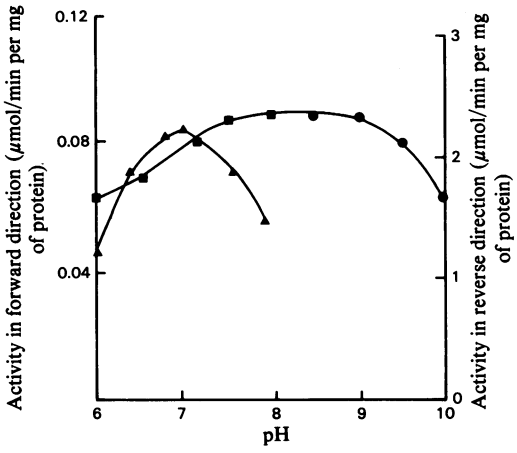


Fig. 2. *pH optima for human brain sorbitol dehydrogenase*

■ and ●, Activity in forward direction (sorbitol→fructose). The assay mixture contained 3mM-NAD^+, 450mM-sorbitol, enzyme and one of the following buffers: 60mM-sodium phosphate/citric acid (pH6.0–8.0) (■) or 30mM-sodium pyrophosphate (pH8.5–10.0) (●). ▲, Activity in reverse direction (fructose→sorbitol). The assay mixture contained 110μM-NADH, 0.27M-fructose, enzyme and 60mM-sodium phosphate/citric acid buffer (pH6.0–8.0).

Substrate specificity

The substrate specificity for human brain sorbitol dehydrogenase is presented in Table 2. With 200μM-NAD^+ as coenzyme the enzyme oxidized a wide range of polyols other than sorbitol.

Although sorbitol is regarded as the principal polyol substrate for the enzyme, xylitol has a K_m value approximately 3-fold lower than that for sorbitol, and exhibits a very similar $V_{max.}$. This pattern of substrate specificity may provide a guide to the possible physiological role of the enzyme under normal conditions for rodent liver. There it has been suggested that the activities of NAD-linked sorbitol dehydrogenase and NAD-linked xylitol dehydrogenase reflect the activity of the same enzyme, namely L-iditol dehydrogenase (Tulsiani & Touster, 1979), which catalyses the oxidation of xylitol to D-xylulose in the glucuronate–xylose cycle. Under these circumstances, sorbitol dehydrogenase, with xylitol as substrate, would be an integral component of this cycle, the first enzyme of which is L-hexonate dehydrogenase, an enzyme that we have previously shown to be present in human brain (O'Brien & Schofield, 1980). However, whether this complete cycle operates in human brain is an aspect open to speculation at this stage.

Table 2. *Substrate specificity of human brain sorbitol dehydrogenase*

Michaelis constants are expressed as means ± S.E.M. as given by computer-fit of the data. Assay mixtures for polyol substrates contained 30mM-sodium pyrophosphate buffer, pH9.0, 200μM-NAD^+, enzyme and various concentrations of polyol. Assay mixtures for ketose substrates contained 150mM-triethanolamine/HCl buffer, pH7.4, 100μM-NADH, enzyme and various concentrations of ketose. The $V_{max.}$ for sorbitol in the forward direction was 0.38μmol of product formed/min per mg of protein.

Substrate	K_m (mM)	$V_{max.}$ (%)
D-Sorbitol	0.45 ± 0.02	100
Galactitol	8.8 ± 0.7	86
D-Mannitol	18 ± 5	75
Xylitol	0.12 ± 0.02	95
Ribitol	1.18 ± 0.07	94
L-Arabitol	25 ± 3	65
D-Fructose	480 ± 40	1350
L-Sorbose	990 ± 80	1080

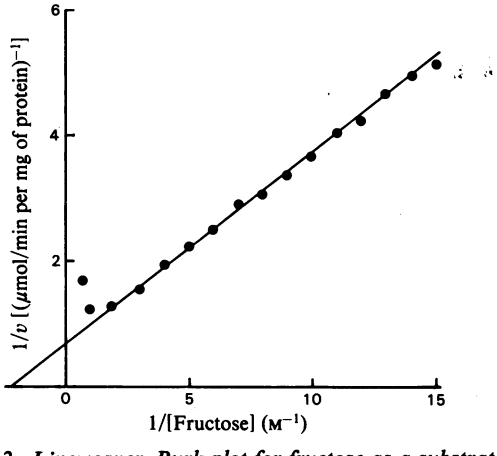


Fig. 3. *Lineweaver–Burk plot for fructose as a substrate of human brain sorbitol dehydrogenase*

Assay mixtures contained 150mM-triethanolamine/HCl buffer, pH7.4, 100μM-NADH, enzyme and various concentrations of fructose in a total volume of 3.0ml. The line was computer-fitted, omitting the data obtained at fructose concentrations above 0.25M to remove interference due to substrate inhibition.

The K_m values for the ketoses were extremely high, being 100–1000 times greater than the concentrations that may be reasonably expected to be the normal physiological range. However, the $V_{max.}$ in the reverse direction with fructose as substrate was more than 10 times greater than the $V_{max.}$ in the forward direction with sorbitol as substrate. The enzyme exhibited substrate inhibition with fructose at concentrations exceeding 0.5M (Fig. 3). Since the

K_m for fructose was 0.48 M, saturating concentrations of fructose could not be used, and the standard assay in fact used a fructose concentration less than the K_m value. Substrate inhibition by fructose may well be a common feature of this enzyme, having been found in the human serum and liver enzyme (Rose & Henderson, 1975).

Substrate specificities have been reported for a number of sorbitol dehydrogenases isolated from a variety of tissues and species. These specificities vary widely, and it is not clear whether the variations are truly due to tissue and species differences or are in fact due to differences in assay concentrations and conditions used by different authors. The K_m values reported here for human brain sorbitol dehydrogenase are in reasonable agreement with those reported for the rat brain enzyme (Rehg & Torack, 1977), with the notable exception of the value of 0.077 M for fructose for the rat enzyme as compared with 0.48 M for the human brain enzyme. Rose & Henderson (1975) reported high K_m values for fructose for human serum and liver sorbitol dehydrogenase (0.35 M and 0.38 M respectively). However, the reported values of 0.040 M for the human lens enzyme (Jedziniak, 1980) and 0.032 M for the calf lens enzyme (Jedziniak *et al.*, 1973) suggest that the lenticular enzymes have a much higher affinity for fructose than do the enzymes from other tissues.

The K_m value for NAD^+ for human brain sorbitol dehydrogenase with sorbitol as the polyol substrate was 11 μM. Activity with $NADP^+$ was negligible. In the reverse direction the K_m value for NADH with fructose as the ketose substrate was 9 μM. As the $V_{max.}$ is far greater in this direction, it was possible to detect activity with NADPH as substrate. However, the K_m for NADPH was 1 mM, and the $V_{max.}$ was only 40% of that obtained with NADH. These K_m values are essentially similar to those reported for the enzyme from human lens (Jedziniak, 1980) and calf lens (Jedziniak *et al.*, 1973). However, values reported for the enzyme from sheep liver (Smith, 1962; Walsall *et al.*, 1978) and rat liver (Walsall *et al.*, 1978) are substantially higher, being in the range 0.17–0.6 mM for NAD^+.

Kinetic mechanism

In order to determine the enzyme mechanism, the enzyme reaction was studied in the reverse direction, since the maximal velocities obtained with fructose and NADH as substrates were very much greater than those in the forward direction. Variation of both fructose and NADH gave intersecting lines on a double-reciprocal plot (Fig. 4), indicating a sequential mechanism. Kinetic constants obtained from these data by computer fit to the rate equation (Cleland, 1979) are presented in Table 3.

To determine the type of sequential mechanism, product-inhibition studies were performed with the reverse reaction. The results are presented in Table 4. Representative double-reciprocal plots are given in Figs. 5 and 6. The results are consistent with either a Random Bi Bi mechanism with two dead-end complexes or an Ordered Bi Bi (Theorell–Chance) mechanism.

In order to differentiate between these alternatives, substrate binding was investigated. Both NADH and fructose protected the enzyme against thermal inactivation at 42°C (Figs. 7*a* and 7*b*). These findings support random rather than ordered binding of substrates, and taken together with the product-inhibition data strongly suggest that the mechanism is Random Bi Bi, with two dead-end complexes. The random mechanism is shown in Scheme 1. This is slightly different from the mechanism reported for the sheep liver enzyme,

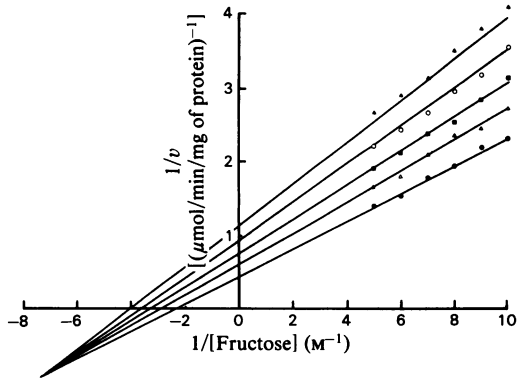


Fig. 4. *Double-reciprocal plot obtained for human brain sorbitol dehydrogenase by varying fructose and NADH concentrations*

Assay mixtures contained 150 mM-triethanolamine/HCl buffer, pH 7.4, enzyme, 0.100 M-, 0.111 M-, 0.125 M-, 0.143 M-, 0.167 M- or 0.200 M-fructose and one of the following concentrations of NADH: 8 μM (▲), 10 μM (○), 14 μM (■), 20 μM (△) or 40 μM (●). Lines are computer-fitted to the rate equation (Cleland, 1979). Constants derived from these data are presented in Table 3.

Table 3. *Kinetic constants of sorbitol dehydrogenase from human brain*

Constants were determined by computer-fitting the data presented in Fig. 4 to the rate equation (Cleland, 1979). Values are means ± S.E.M.

$V_{max.}$ =	4.1 ± 1.4 μmol/min per mg of protein
K_m (NADH) =	28.6 ± 1.4 μM
K_m (fructose) =	0.69 ± 0.28 M
K_i (NADH) =	5.6 ± 1.7 μM
K_i (fructose) =	0.134 ± 0.058 M

Table 4. *Product-inhibition pattern for human brain sorbitol dehydrogenase (non-physiological direction)*
Constants are expressed as means ± S.E.M. and are as determined by computer-fit to the rate equations (Cleland, 1979). Assay mixtures contained 150mM-triethanolamine/HCl buffer, pH 7.4, plus 200mM-fructose when the NADH concentration was varied, or plus 10μM-NADH when the fructose concentration was varied, enzyme and inhibitor.

Substrate with varied concentration	Substrate with fixed concentration	Inhibitor	Inhibition type	Inhibition constants (mM) K_{IS}	Inhibition constants (mM) K_{II}
NADH	Fructose	Sorbitol	Non-competitive	510 ± 90	250 ± 20
NADH	Fructose	NAD^+	Competitive	0.26 ± 0.01	—
Fructose	NADH	Sorbitol	Competitive	150 ± 10	—
Fructose	NADH	NAD^+	Non-competitive	0.31 ± 0.04	0.96 ± 0.16

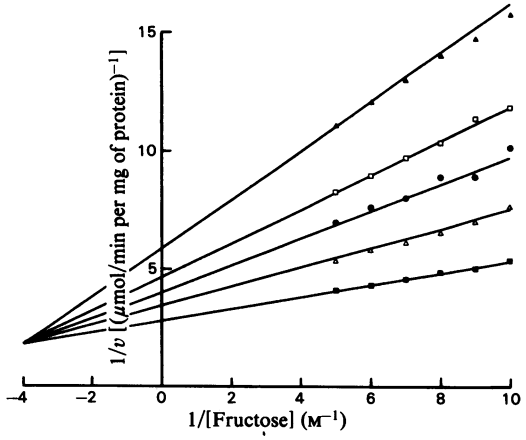


Fig. 5. *Inhibition of human brain sorbitol dehydrogenase by NAD^+ with fructose as the varied substrate*
Assay mixtures contained 150mM-triethanolamine/HCl buffer, pH 7.4, 20μM-NADH (unsaturating concentration), enzyme, 0.100M-, 0.111M-, 0.125M-, 0.143M-, 0.167M- or 0.200M-fructose and one of the following concentrations of NAD^+: 0 (■), 0.2mM (△), 0.4mM (●), 0.6mM (□) or 1.0mM (▲). Lines are computer-fitted to the rate equation (Cleland, 1979).

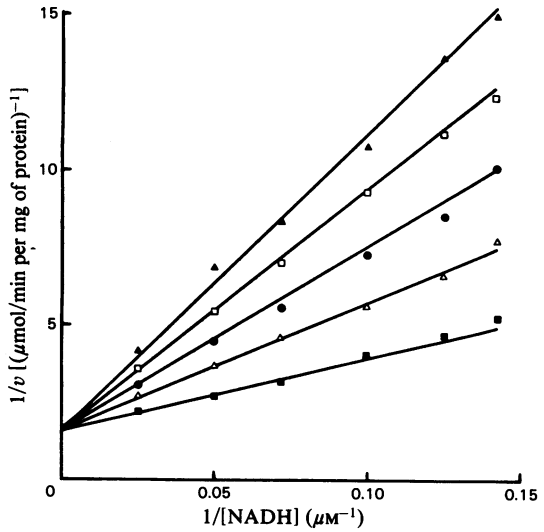


Fig. 6. *Inhibition of human brain sorbitol dehydrogenase by NAD^+ with NADH as the varied substrate*
Assay mixtures contained 150mM-triethanolamine/HCl buffer, pH 7.4, 200mM-fructose, enzyme, 7μM-, 8μM-, 10μM-, 14μM-, 20μM- or 40μM-NADH and one of the following concentrations of NAD^+: 0 (■), 0.2mM (△), 0.4mM (●), 0.6mM (□) or 0.8mM (▲). Lines are computer-fitted to the rate equation (Cleland, 1979).

namely a random mechanism in which only the enzyme–sorbitol–NADH dead-end complex is formed (Christensen *et al.*, 1975).

Evidence for an essential thiol group and involvement of a metal ion

The human brain enzyme was inhibited by a number of thiol-blocking reagents. *p*-Chloromercuribenzoate (5μM) gave 90% inhibition in 10min at 30°C, which is in agreement with the complete inhibition of the sheep liver enzyme by 0.1mM-*p*-chloromercuribenzoate reported by Smith (1962). Similarly, incubation of the enzyme for 30min with 0.1mM-5,5′-dithiobis-(2-nitrobenzoate), 1mM-iodoacetate and 1mM-iodoacetamide resulted in 27%, 53% and 20% inhibition respectively. NADH protected against inhibition by 7.5mM-iodoacetate. Fructose gave no protection. The enzyme was also inhibited by incubation with cysteine, but this inhibition was highly pH-dependent. Inhibition was observed only above pH 8.0; at pH 9.0 incubation of the enzyme with 2.5mM-cysteine for 20min at 30°C gave 80% inhibition. Similar inhibition by cysteine was observed for the rat brain enzyme (Rehg & Torack, 1977).

Human brain sorbitol dehydrogenase was inhibited by 50% by 1.2mM-*o*-phenanthroline, this in-

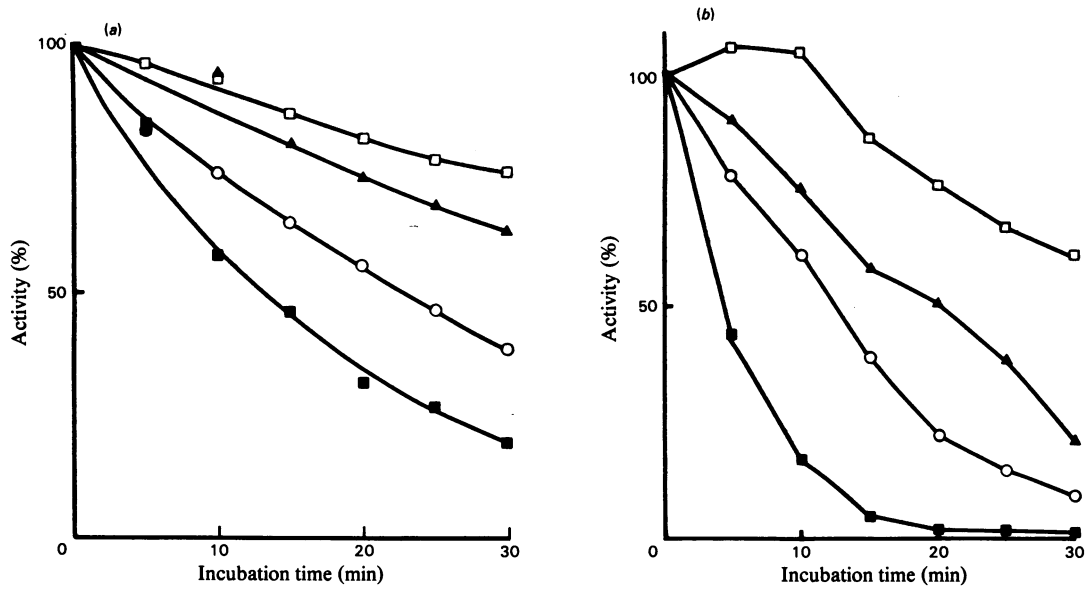
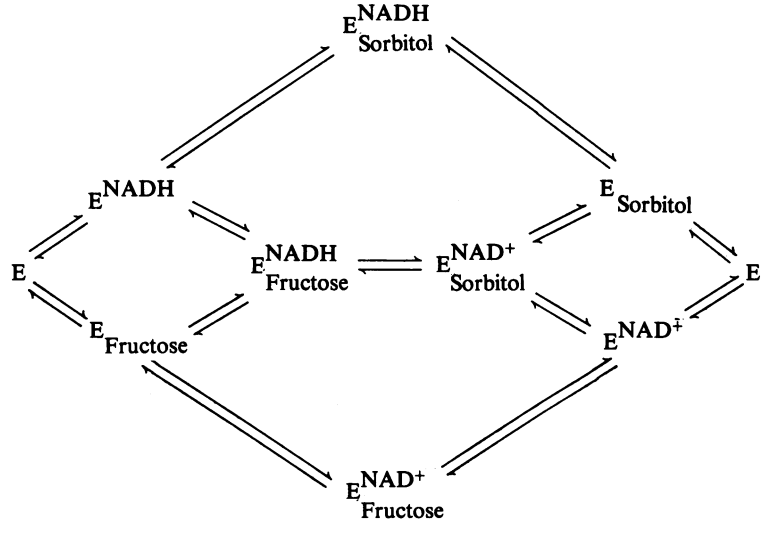


Fig. 7. *Thermal inactivation of human brain sorbitol dehydrogenase*

(*a*) Protection by NADH. Enzyme was incubated at 42°C in the absence of fructose but with the indicated concentrations of NADH, and samples were assayed at 5min intervals. Assay mixtures contained 150mM-triethanolamine/HCl buffer, pH 7.4, 200mM-fructose, 100μM-NADH and enzyme in a total volume of 3.0ml. The concentrations of NADH were: 0 (■), 0.5μM (○), 2μM (▲) and 3μM (□). (*b*) Protection by fructose. Enzyme was incubated in the absence of NADH but with the indicated concentrations of fructose. Experimental conditions were as above. The concentrations of fructose were: 0 (■), 0.05M (○), 0.1M (▲) and 1M (□).



Scheme 1

hibition being similar to that reported for the sheep liver enzyme (Smith, 1962). EDTA gave time-dependent inhibition; incubation of the enzyme with 1mM-EDTA at 30°C resulted in complete loss of activity within 15min. Rehg & Torack (1977) reported inhibition of the rat brain enzyme by EDTA and reversal of that inhibition by 3.5mM-$ZnSO_4$. However, similar reversal of the EDTA

inhibition was not observed for the human brain enzyme. In fact, the addition of 3.5 mM-$ZnSO_4$ itself caused immediate and complete inhibition.

The totality of these inhibition studies suggests that the human brain enzyme, in common with other sorbitol dehydrogenases, contains thiol groups necessary for catalytic activity, and also that the enzyme may be dependent on a metal ion for full activity. The essential role of thiol groups has been more fully substantiated by studies on the sheep liver enzyme with carboxymethylation techniques, which suggested the presence of a reactive cysteine residue close to the active site (Jeffrey *et al.*, 1981).

Inhibition by quercetin

Another, quite different, compound that was tested as a possible inhibitor of human brain sorbitol dehydrogenase was the flavonoid quercetin. This flavone derivative has been shown to be a strong inhibitor of the reducing enzymes of the polyol pathway in human brain, namely aldose reductase and hexonate dehydrogenase (M. M. O'Brien, P. S. Schofield & M. R. Edwards, unpublished work), as well as an inhibitor of lens aldose reductase from a number of species (Varma *et al.*, 1975; Varma & Kinoshita, 1976). In relation to the polyol pathway it is generally thought that quercetin is a specific aldose reductase inhibitor. However, this is clearly not the case for the human brain enzymes; the brain sorbitol dehydrogenase was inhibited by 50% by 45 μM-quercetin. Such inhibition of sorbitol dehydrogenase from any source has not previously been reported.

Clinical significance of the properties of the enzyme

From the metabolic and clinical aspects, the two major polyol substrates are sorbitol and galactitol, the former assuming importance in diabetes, and the latter in complications associated with galactosaemia. The potential of the human brain to oxidize intracellularly synthesized galactitol is clearly considerably less than that for sorbitol. The K_m of almost 10 mM for galactitol (compared with less than 1 mM for sorbitol), together with a potential maximal catalytic activity less than that for sorbitol, suggest that the enzyme is incapable of oxidizing any considerable quantity of galactitol. It thus follows that markedly elevated concentrations of galactitol would be expected in brain tissue in galactosaemia. Values for cerebral galactitol concentrations are not freely available for the human, but are available for rat brain. In this latter case the predicted elevated concentrations of galactitol are indeed found in galactosaemia (namely 9.3 mmol/kg net wt. compared with 0.04 mmol/kg net wt. in the controls). Since the properties of human and rat brain enzymes are similar, this lends support to the suggestion that, in the case of galactitol, flux through sorbitol dehydrogenase is, in fact, severely limited by the substrate specificity of the enzyme. On the other hand, the concentration of sorbitol, the polyol that can be readily oxidized by the brain sorbitol dehydrogenase, is only marginally elevated in the brains of diabetic rats. The concentration of 0.09 mmol/kg net wt. is only 0.1% of that of the corresponding galactosaemic polyol, and is only about half the concentration of the product, fructose (0.15 mmol/kg net wt.).

The significance of these values is twofold, namely (*a*) that cerebral galactitol accumulation is a major complication of galactosaemia, and (*b*) that cerebral sorbitol accumulation, and hence possible tissue damage, is negligible in diabetes and that both these consequences arise by virtue of the substrate specificity of the sorbitol dehydrogenase. (Although other factors such as $[NAD^+]/[NADH]$ ratios will also affect the flux through the enzyme, they are unlikely to overcome these constraints imposed by the enzyme specificity.)

The proposition that brain polyol synthesis is not a major problem in diabetes runs counter to the proposed role of sorbitol in diabetic complications, especially in cataractogenesis, but particularly in cerebral oedema. However, it may be that a re-assessment of the role of sorbitol in human diabetes, and hence of the aldose reductase–sorbitol dehydrogenase system, is required. This has been highlighted by the studies by Jedziniak *et al.* (1981). Whereas it has been tacitly assumed that the human lens behaves in a similar manner to animal lens models in diabetes, these authors have described, for the first time, fundamental differences in the pattern of these enzymes in human and animal lens. They suggest that 'the high level of polyol dehydrogenase relative to aldose reductase in human lens may account for the observations that considerable polyol is in the fructose form in human lens whereas by virtue of low polyol dehydrogenase level sorbitol is the predominant product of the sorbitol pathway in the animal lens'. The clear inference of those studies, and of the properties of the brain enzyme reported in the present paper, is that in human lens, and probably human brain, there is no significant accumulation of polyol in diabetes, and, as a result, no significant osmotic imbalance. Consequently the proposition that polyol accumulation in brain is responsible for the development of severe cerebral oedema would no longer be valid, except possibly under unusual circumstances. Indeed, the fact that some studies have shown that in hyperglycaemia the concentrations of brain sorbitol are of virtually no osmotic significance (Arieff & Kleeman, 1973) is consistent with this position, which is the direct consequence of the properties described above for the brain sorbitol dehydrogenase.

This work was supported in part by an Australian Research Grants Committee Grant to P. J. S.

References

Arieff, A. I. & Kleeman, C. R. (1973) *J. Clin. Invest.* **52**, 571–583

Barretto, O. C. O. & Beutler, E. (1975) *J. Lab. Clin. Med.* **85**, 645–649

Chrambach, A., Reisfeld, R. A., Zacchari, J. & Wyckoff, M. (1967) *Anal. Biochem.* **20**, 150–154

Christensen, V., Tuchsen, E. & Anderson, B. (1975) *Acta Chem. Scand.* **29**, 81–87

Cleland, W. W. (1979) *Methods Enzymol.* **63**, 103–138

Clements, R. S., Prockop, L. D. & Winegrad, A. I. (1968) *Lancet* **ii**, 384–386

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427

Itzhaki, R. F. & Gill, D. M. (1964) *Anal. Biochem.* **9**, 401–410

Jedziniak, J. A. (1980) *Invest. Ophthalmol. Visual Sci.* (*ARVO Abstr.*) 11

Jedziniak, J. A., Yates, E. M. & Kinoshita, J. H. (1973) *Exp. Eye Res.* **16**, 95–104

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

Jeffrey, J., Cummins, L., Carlquist, M. & Jörnvall, H. (1981) *Eur. J. Biochem.* **120**, 229–234

Leissing, N. & McGuinness, E. T. (1978) *Biochim. Biophys. Acta* **524**, 254–261

O'Brien, M. M. & Schofield, P. J. (1980) *Biochem. J.* **187**, 21–30

Prockop, L. D. (1971) *Arch. Neurol.* (*Chicago*) **25**, 126–140

Rehg, J. E. & Torack, R. M. (1977) *J. Neurochem.* **28**, 655–660

Rose, C. I. & Henderson, A. R. (1975) *Clin. Chem.* **21**, 1619–1626

Smith, M. G. (1962) *Biochem. J.* **83**, 135–144

Tulsiani, D. R. P. & Touster, O. (1979) *Diabetes* **28**, 793–798

Varma, S. D. & Kinoshita, J. H. (1976) *Biochem. Pharmacol.* **25**, 2505–2513

Varma, S. D., Mikuni, I. & Kinoshita, J. H. (1975) *Science* **188**, 1215–1216

Walsall, E. P., Lyons, S. A. & Metzger, R. P. (1978) *Comp. Biochem. Physiol. B* **59**, 213–218

Winegrad, A. I., Clements, R. S. & Morrison, A. D. (1972) *Handb. Physiol. Sect. 7* **1**, 457–471