Polyol-Pathway Enzymes of Human Brain

PARTIAL PURIFICATION AND PROPERTIES OF ALDOSE REDUCTASE AND HEXONATE DEHYDROGENASE

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Aldose reductase and hexonate dehydrogenase were isolated from human brain and partially purified. The two enzymes exhibited distinctive substrate-specificity profiles with a variety of aldoses, and aliphatic and aromatic aldehydes. Aldose reductase exhibited a high affinity for DL-glyceraldehyde (K_m of $62\mu\text{M}$) and a low affinity (K_m of 90mM) for glucose, the physiological substrate of the polyol pathway. Hexonate dehydrogenase exhibited a relatively low affinity for D-glucuronate (K_m of 4.6mM) and a very low affinity for glucose (K_m of 390mM). Both enzymes exhibited a high specificity for NADPH, and both were inhibited competitively by NADP^+ . Hexonate dehydrogenase was inhibited by iodoacetate, iodoacetamide, *N*-ethylmaleimide and *p*-chloromercuribenzoate. Preincubation with 2-mercaptoethanol resulted in activation. Both enzymes were inhibited by a number of barbiturates (barbital, phenobarbital and pentobarbital) and by the central-nervous-system drugs diphenylhydantoin and ethosuccinimide. The substrate specificity and pattern of inhibition suggest that the two enzymes isolated correspond to two of four previously reported aldehyde reductases isolated from human brain.

The polyol pathway is a minor pathway of glucose metabolism and consists of two successive dehydrogenases, namely aldose reductase and sorbitol dehydrogenase, which catalyse the following reactions.

Under normal conditions, there is little flux through the pathway in most tissues. However, in diabetic hyperglycaemic conditions, the flux through the pathway increases dramatically, leading to increased intracellular concentrations of sorbitol. Because of the impermeability of most cell membranes to sorbitol, the accumulation of this metabolite establishes an osmotic imbalance that ultimately causes the development of a number of pathological conditions in various tissues. In the lens, the sorbitol accumulation is responsible for the development of diabetic cataractogenesis and in peripheral nerve it has been implicated in the development of diabetic neuropathy. In the brain, it has been suggested to be the causative agent in the development of fatal cerebral oedema.

The first enzyme, aldose reductase (alditol-NADP⁺ oxidoreductase, EC 1.1.1.21), is NADPH-specific and exhibits a broad substrate specificity, reducing a number of aldoses and aldehydes to their

corresponding polyols and alcohols. It was first demonstrated in extracts of sheep seminal vesicles by Hers (1957). The presence of the enzyme in rat lens was established soon after (van Heyningen, 1959) and it was subsequently studied in detail in lens from a number of animals, including rat (Kinoshita *et al.*, 1968; Varma & Kinoshita, 1974), rabbit (Kinoshita *et al.*, 1963; Hayman *et al.*, 1966; Chylack & Kinoshita, 1969) and calf (Kinoshita *et al.*, 1963; Hayman & Kinoshita, 1965; Jedziniak & Kinoshita, 1971). Bovine lens aldose reductase has been purified to homogeneity (Sheaff & Doughty, 1976).

Demonstration of the enzyme in brain tissue was achieved by Moonsammy & Stewart (1967), who

purified the aldose reductase from ox brain. However, these authors concomitantly demonstrated the existence of a second NADPH-specific aldose-reducing enzyme, namely hexonate dehydrogenase, in the brain tissue. Subsequently both aldose reductase and hexonate dehydrogenase have been purified from calf brain (Dons & Doughty, 1976).

The reason for the presence of both aldose reductase and hexonate dehydrogenase, in tissues other than liver and seminal vesicles, is unclear. Aldose reductase in lens (and, by extrapolation, in the brain) has been suggested to function in a transhydrogenase system, or to be purely vestigial. A similar situation exists for hexonate dehydrogenase, whose primary role in non-primates is in the hepatic synthesis of ascorbic acid, in which it is responsible for the reduction of D-glucuronate to L-gulonate.

The only evidence to date for the presence of aldose reductase and hexonate dehydrogenase in human brain, and hence operation of the polyol pathway in this tissue, is indirect. For instance, the presence of fructose in the cerebrospinal fluid of severely hyperglycaemic diabetic subjects has been observed (J. C. Hutton & P. J. Schofield, unpublished work). Hence, a fundamental question is whether the human brain contains both aldose reductase and hexonate dehydrogenase, and if so, what are the characteristic properties of these enzymes.

Generically, both aldose reductase and hexonate dehydrogenase are NADPH-specific aldehyde reductases. In neural tissues, particularly brain, such aldehyde reductases have been extensively studied in respect of their role in the metabolism of biogenic aldehydes. For the human brain, Ris & von Wartburg (1973) have reported the presence of a number of 'multiple forms' of aldehyde reductase. In view of these findings, a second fundamental question is whether any of the 'multiple forms' of aldehyde reductase are, in fact, aldose reductase and hexonate dehydrogenase.

The present paper demonstrates the existence of aldose reductase and hexonate dehydrogenase in human brain, and describes their separation and partial purification. Limited studies of substrate specificity and of inhibition by a number of compounds known to affect neural function allows comparison with the 'multiple forms' of aldehyde reductase, as described by Ris & von Wartburg (1973).

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% (w/v) NaCl and stored at -20°C until required.

Blue Dextran, bovine serum albumin, cytochrome *c*, yeast alcohol dehydrogenase (EC 1.1.1.1), *p*-nitrobenzaldehyde, *p*-nitrobenzyl alcohol and pyridine-3-aldehyde were obtained from Sigma Chemical Company, St. Louis, MO, U.S.A. *m*-Nitrobenzaldehyde was purchased from Fluka, Buchs, Switzerland, and 4-chlorobenzaldehyde was from Hopkin and Williams, Chadwell Heath, Essex, U.K. NADP⁺ was obtained from Calbiochem (Australia) Pty., Sydney, Australia, and NADPH was from Boehringer Mannheim (Australia) Pty., Sydney, Australia.

DEAE-cellulose (DE-52) was obtained from Whatman, Maidstone, Kent, U.K. Sephadex G-100, purchased from Pharmacia Fine Chemicals, Uppsala, Sweden, was pre-swollen at 100°C for 6 h before use in 10 mM-Tris/HCl, pH 6.8, containing 5 mM-2-mercaptoethanol.

Sodium barbitone was obtained from BDH, Poole, Dorset, U.K., ethosuccinimide (3-ethyl-3-methylpyrrolidine-2,5-dione) and sodium diphenylhydantoin (sodium 5,5-diphenylimidazolidine-2,4-dione) were gifts from Parke-Davis and Co., Carringbah, N.S.W., Australia; phenobarbitone sodium was from May and Baker (Australia) Pty., Waterloo, N.S.W., Australia, and pentobarbitone sodium was from Abbott Laboratories Pty., Kurnell, N.S.W., Australia.

Substrates and inhibitors that were not water soluble were dissolved in absolute ethanol. The maximum final concentration of ethanol in the enzyme assay was 3.3% (v/v).

Methods

Enzyme assay

Activity of hexonate dehydrogenase was determined by measurement of the decrease in absorbance at 340 nm as NADPH was oxidized. The assay mixture contained 100 mM-sodium phosphate, pH 6.2, 120 μM -NADPH, 20 mM-D-glucuronate and enzyme in a total volume of 3.0 ml. The reaction was initiated by addition of the D-glucuronate. An enzyme unit was defined as the amount of enzyme oxidizing 1 μmol of NADPH per minute at 30°C under the assay conditions. Linearity was observed for at least 3 min after initiation of the reaction, and velocity was directly proportional to protein concentration.

Activity of aldose reductase was measured in the same way except that 1 mM-glyceraldehyde was substituted for the 20 mM-D-glucuronate.

Ethanol had no effect on the rate of reaction with either enzyme at the concentrations added to some assays.

Protein determination

Protein concentrations of partially purified extracts were determined by the microbiuret method of

Itzhaki & Gill (1964). Fractions obtained by column chromatography were scanned for protein by measurement of the absorbance at 280 nm, or by the method of Warburg & Christian (1941). The protein concentrations of enzyme samples at later stages of the purification procedure were determined by the method of Lowry *et al.* (1951). Bovine serum albumin was used as a protein standard in all protein assays.

Polyacrylamide-gel electrophoresis

Polyacrylamide gels (7%) were prepared as described by Davis (1964). The marker dye was 0.01% (w/v) Bromophenol Blue and the samples were applied in 20% (w/v) sucrose.

The method of Chrambach *et al.* (1967) was employed to observe protein migration. Enzyme activity was detected by a modification of the procedure described by Sheaff & Doughty (1976). Gels were immersed in 63 mM-sodium phosphate, pH 7.0, for 10 min and then incubated in the reaction mixture for 30 min in the dark at 37°C. The reaction mixture contained 5 mg of *p*-nitrobenzyl alcohol/ml, 1 mg of NADP⁺/ml, 63 mM-sodium phosphate, pH 7.0, 0.02 mg of phenazine methosulphate/ml and 0.35 mg of Tetrazolium Violet/ml. Enzyme activity was indicated by the development of a fluorescent pink band.

Purification procedure (modified from Dons & Doughty, 1976)

All buffers contained 5 mM-2-mercaptoethanol and all steps were performed at 0–4°C.

1. *Preparation of crude extract.* Approx. 100 g of human brain (fresh or frozen) was homogenized by motor driven pestle in 2 ml of 20 mM-sodium phosphate (pH 7.4)/g of tissue. The homogenate was centrifuged at 30 000 g for 30 min 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 (1 mg of protamine sulphate/20 mg of protein) over a period of 15 min. The pH was monitored and maintained at pH 7.0 with 0.1 M-NaOH. After an additional 5 min stirring, the precipitate was removed by centrifugation at 23 000 g for 15 min and discarded.

3. *(NH₄)₂SO₄ precipitation.* Solid (NH₄)₂SO₄ (242 g/litre) was slowly added with stirring to the supernatant from the previous step until 40% saturation was attained. After a further 15 min stirring, the suspension was centrifuged at 23 000 g for 10 min and the precipitate was discarded. The supernatant was brought slowly to 60% saturation with the appropriate mass of solid (NH₄)₂SO₄ (131 g/litre), stirred for an additional 30 min and re-centrifuged. The resulting precipitate was dissolved in 6 ml of 10 mM-sodium phosphate, pH 7.2,

and dialysed overnight against 100 vol. of the same buffer.

4. *DEAE-cellulose chromatography.* The dialysed solution was applied to a DEAE-cellulose column (1.5 cm × 25 cm) that had been previously equilibrated with 10 mM-sodium phosphate, pH 7.2. This same buffer was washed through the column until the absorbance of the eluate at 280 nm was less than 0.010. A 300 ml linear 10–60 mM-sodium phosphate gradient, pH 7.2, was used to elute the enzymes. The flow rate was 40 ml/h and 5 ml fractions were collected. The enzyme activities were concentrated by a 0–80% (560 g/litre) (NH₄)₂SO₄ precipitation.

As the yield of aldose reductase at this stage was extremely low, further purification was carried out on hexonate dehydrogenase only.

A second DEAE-cellulose column (0.75 cm × 15 cm) was equilibrated to pH 7.2 with 10 mM-sodium phosphate, pH 7.2. The sample was applied and the column was washed with 20 ml of the above buffer. A linear 0–0.2 M-(NH₄)₂SO₄ gradient in 10 mM-sodium phosphate, pH 7.2, was used to elute the enzyme activity. The flow rate was 20 ml/h and the fraction volume was 2 ml. Active fractions were again concentrated by 0–80% (560 g/litre) (NH₄)₂SO₄ precipitation.

5. *Sephadex G-100 chromatography.* The concentrated enzyme preparation was applied to a Sephadex G-100 column (1.5 cm × 90 cm), which was equilibrated and eluted with 10 mM-Tris/HCl, pH 6.8. A pressure head of 10 cm was utilized, giving a flow rate of 2.6 ml/h. A 0–85% (610 g/litre) (NH₄)₂SO₄ precipitation was used to concentrate fractions containing enzyme activity.

Results and Discussion

Aldose reductase was shown to be present in the human brain at a level of 40 m-units/g wet wt. of tissue. The enzyme was clearly separated from hexonate dehydrogenase by the use of a phosphate gradient on DEAE-cellulose. Thus both aldose reductase and hexonate dehydrogenase, both of which are NADPH-specific aldose-reducing enzymes, are present in human brain. In fact, screening of the column eluent with 1 mM-*p*-nitrobenzaldehyde as substrate demonstrated the existence of a third NADPH-specific reductase, which eluted before the application of the phosphate gradient. This latter enzyme activity appeared to be the general aldehyde reductase of biogenic aldehyde metabolism, and exhibited no activity with glyceraldehyde and glucuronate as substrate at the concentrations tested. It is thus not related to polyol-pathway metabolism, and quite distinct from the two polyol-pathway reductases. No further studies were carried out on this enzyme fraction.

By the combination of protamine sulphate treat-

ment, $(\text{NH}_4)_2\text{SO}_4$ precipitation (40–60% saturation) and elution from DEAE-cellulose with 10–60 mM-sodium phosphate gradient (pH 7.2), the human brain aldose reductase was purified 88-fold, with 13% yield (Fig. 1). Treatment of the brain homogenate with detergent [1% (v/v) Triton X-100] did not increase the yield of enzyme activity at that stage. The yield of enzyme was identical for both fresh human brain samples and those that had been stored at -20°C for up to 6 months. Polyacrylamide-gel electrophoresis demonstrated that the partially purified aldose reductase was the only NADPH-specific aldehyde-reducing enzyme species present. When maintained as a suspension in 20%

(w/v) $(\text{NH}_4)_2\text{SO}_4$, 10 mM-sodium phosphate buffer, pH 7.2, containing 5 mM-2-mercaptoethanol, the enzyme lost little activity when stored at -20°C for 4 weeks, whereas storage at 4°C for 3 weeks resulted in 50% loss of activity. Addition of solid poly(ethylene glycol) to the suspension to achieve a final concentration of 1% (w/v) did not increase the stability of the enzyme at either temperature of storage; Sheaff & Doughty (1976) had reported stabilization of the bovine lens aldose reductase by 1% (w/v) poly(ethylene glycol) for storage at -60°C .

The pH optimum, with glyceraldehyde as substrate, was 6.6 (Fig. 2a). This value was also

Fig. 1. Separation of human brain aldose reductase (Δ) and hexonate dehydrogenase (O) with a DEAE-cellulose column (1.5 cm $\times$ 25 cm) at pH 7.2

---- indicates the sodium phosphate concentration. The flow rate was 40 ml/h and 5 ml fractions were collected. Protein concentration ($\bullet$) was determined by the method of Warburg & Christian (1941). Aldose reductase assays contained 100 mM- NaH_2PO_4 , pH 6.2, 120 μM -NADPH, 1.0 mM-glyceraldehyde and enzyme sample in a 3 ml volume. Hexonate dehydrogenase assays contained 100 mM-sodium phosphate, pH 6.2, 120 μM -NADPH, 20 mM-glucuronate and enzyme in a 3 ml volume.

Fig. 2. pH optima for human brain aldose reductase and hexonate dehydrogenase

(a) Aldose reductase. Assays contained enzyme, 120 μM -NADPH, 1 mM-glyceraldehyde and either 60 mM-sodium phosphate/citric acid buffer (pH 4.5–6.0), 60 mM-sodium phosphate buffer (pH 6.5–8.0) or 30 mM-sodium pyrophosphate buffer (pH 8.0–10.0). (b) Hexonate dehydrogenase. Assays contained enzyme, 120 μM -NADPH, 20 mM-glucuronate and one of the following buffers: 60 mM-sodium phosphate/citric acid, pH 5.0–7.0 (O); 60 mM-sodium phosphate, pH 6.2–7.5 (Δ); 30 mM-sodium pyrophosphate, pH 8.0–9.5 ($\blacktriangle$); 100 mM-imidazole/HCl, pH 6.2–7.8 ($\blacksquare$). Measurement of pH in the cuvette was made before and after assay.

obtained for the aldose reductase from ox brain (Moonsammy & Stewart, 1967) and from calf brain (Dons & Doughty, 1976). Glyceraldehyde has normally been used as the substrate for monitoring aldose reductase activity.

Studies of the coenzyme specificity of the aldose reductase indicated a high affinity for NADPH, the K_m being $3.5\mu\text{M}$. This value is similar to those obtained for bovine lens aldose reductase ($6\mu\text{M}$) (Hayman & Kinoshita, 1965) and for the ox brain enzyme ($1.2\mu\text{M}$) (Moonsammy & Stewart, 1967). The activity with $120\mu\text{M}$ -NADH was only 13% of that with $120\mu\text{M}$ -NADPH; this finding is in agreement with the value of 10–15% obtained for the bovine lens aldose reductase (Hayman & Kinoshita, 1965), and of 20% for the calf brain enzyme (Dons & Doughty, 1976). NADP^+ was a competitive inhibitor with a K_i of $9\mu\text{M}$, which is similar to that obtained by Sheaff & Doughty (1976) for the pure aldose reductase from bovine lens ($8\mu\text{M}$).

Aldose reductase from a number of non-primate tissues is characterized by a high affinity for aliphatic aldehydes and a relatively high affinity for aldoses. The brain enzyme exhibited this pattern of specificity (Table 1) with the K_m for glyceraldehyde being $62\mu\text{M}$, which is similar to the value of $110\mu\text{M}$ for the ox brain enzyme (Moonsammy & Stewart, 1967) and $30\mu\text{M}$ for bovine lens enzyme (Sheaff & Doughty, 1976). For aldoses such as glucose, the human enzyme had a similar K_m (90mM) to that of 70mM found for the bovine lens. In general, the substrate specificity of the human brain enzyme was similar to that of the bovine lens and bovine brain enzymes. However, detailed comparison of the substrate specificity of the human brain enzyme with that from various other mammalian tissues is handicapped due to the limited number of common substrates used in the various studies.

Glycerol was shown to be a competitive inhibitor with respect to glyceraldehyde, with a K_i of 230mM (Fig. 3). Inhibition of aldose reductase by glycerol has not been previously reported, possibly because of the high concentrations of glycerol required to demonstrate such inhibition; for the bovine lens enzyme, Jedziniak & Kinoshita (1971) stated that

Fig. 3. Product inhibition of human brain aldose reductase by glycerol with glyceraldehyde as the variable substrate

Assays contained 100mM-sodium phosphate, pH 6.2, $120\mu\text{M}$ -NADPH, enzyme, glyceraldehyde at the indicated concentrations and one of the following glycerol concentrations: 0 (Δ); 0.1 M ($\bullet$); 0.2 M ($\square$); 0.3 M ($\blacktriangle$). The reaction was initiated by addition of the glyceraldehyde.

Table 1. Substrate specificities and $V_{\max}$ values for human brain aldose reductase and hexonate dehydrogenase. Assays (performed at 30°C) contained 100mM-sodium phosphate, pH 6.2, $120\mu\text{M}$ -NADPH, enzyme sample and various concentrations of each substrate. Substrate addition initiated the reaction. Values were obtained by Lineweaver-Burk plots with computer-fitted lines. $V_{\max}$ values are expressed as a percentage of that obtained for *p*-nitrobenzaldehyde. —, value not determined.

Substrate	Aldose reductase		Hexonate dehydrogenase	
	K_m (mM)	$V_{\max}$	K_m (mM)	$V_{\max}$
<i>p</i> -Nitrobenzaldehyde	0.011	100	0.145	100
Pyridine-3-aldehyde	—	—	1.2	102
<i>m</i> -Nitrobenzaldehyde	—	—	2.4	47
Benzaldehyde	—	—	3.0	21
D-Glucuronate	33	45	4.6	97
DL-Glyceraldehyde	0.062	73	5.0	47
4-Chlorobenzaldehyde	—	—	5.6	70
D-Xylose	16.3	60	260	11
D-Glucose	90	23	390	0.9

'addition of various levels of glycerol did not affect the forward reaction'.

Hexonate dehydrogenase was first isolated from rat liver (Mano *et al.*, 1961). A significant role for this enzyme in non-primates is in the reduction of D-glucuronate to L-gulonate in the biosynthesis of ascorbic acid. Clements *et al.* (1969) have demonstrated the presence of the enzyme in a number of rat and rabbit tissues, including liver, spinal cord and brain, and its absence in other tissues, notably lens.

The activity of hexonate dehydrogenase in human brain was 100munits/g wet wt. of tissue. This value was the same for both fresh tissue and tissue stored at -20°C for up to 6 months. The enzyme was purified 1400-fold with a 7% yield, by a combination of DEAE-cellulose and Sephadex G-100 chromatographic procedures (Table 2). Moon-sammy & Stewart (1967) achieved only 100-fold purification of ox brain hexonate dehydrogenase by using a similar procedure. With a further purification step of isoelectric focusing, Dons & Doughty (1976) achieved an overall 650-fold purification for calf brain hexonate dehydrogenase.

Polyacrylamide-gel electrophoresis was carried out on samples from successive stages of purification and gels were stained for protein and enzyme activity. The final preparation of hexonate dehydrogenase contained one major protein band and four minor bands (Fig. 4). Staining for enzyme activity showed only one NADPH-dependent aldehyde-reducing band to be present after the first DEAE-cellulose step, whereas prior fractions showed two or more bands of activity. The enzyme activity in the final preparation of hexonate dehydrogenase corresponds to the major protein band. In an approx. 20% (w/v) (NH₄)₂SO₄ suspension in 10mM-sodium phosphate buffer, pH 7.2, containing 5mM-2-mercaptoethanol, the enzyme retained full

activity for at least 2 months when stored at -20°C. The enzyme was less stable in dilute solution, which necessitated concentration of enzyme preparations as rapidly as possible. A mol.wt. of 36000 for

Fig. 4. Protein-stained polyacrylamide gels showing successive stages of purification of human brain hexonate dehydrogenase

Gels contained: 50µg of crude extract (i); 50µg of protamine sulphate fraction (ii); 50µg of 40-60% (NH₄)₂SO₄ fraction (iii); 50µg of concentrated sample from the first DEAE-cellulose chromatography step (iv); 20µg of concentrated sample from the Sephadex G-100 column (v). The method is described in the text.

Table 2. Purification of hexonate dehydrogenase from human brain

Purification step	Volume (ml)	Protein concentration (mg/ml)	Total protein (mg)	Specific activity (m-units/mg of protein)	Total activity (m-units)	Yield (%)	Purification (-fold)
1. Crude extract	169	7.60	1280	7.05	9050	100	1
2. Protamine sulphate precipitation	170	5.20	884	15.1	13 380	150	2.1
3. 40-60% (NH ₄) ₂ SO ₄ fraction	9.2	29.0	267	31.3	8360	92	4.4
4. DEAE-cellulose column eluate, phosphate gradient	31.0	0.28	8.7	621	5400	60	88
5. 0-80% (NH ₄) ₂ SO ₄ fraction	1.55	4.8	7.4	553	4100	45	78
6. DEAE-cellulose column eluate, (NH ₄) ₂ SO ₄ gradient	5.7	0.42	2.39	1375	3290	36	195
7. Sephadex G-100	0.25	0.25	0.06	10 160	635	7	1440

human brain hexonate dehydrogenase was obtained by gel filtration after calibration of a Sephadex G-100 column with a number of marker proteins (cytochrome *c*, 12 500; ovalbumin, 45 000; bovine serum albumin, 67 000; yeast alcohol dehydrogenase, 150 000). This molecular weight was similar to the value of 33 000 obtained for the ox brain enzyme (Moonsammy & Stewart, 1967), and identical with that obtained for one of the aldehyde reductases ('low- K_m reductase') from pig brain (Turner & Tipton, 1972).

The pH optimum for hexonate dehydrogenase appeared to be 6.0, but an exact value was difficult to obtain (Fig. 2*b*). Phosphate buffer in the pH range 6.2–7.5 showed an anomalous effect similar to that described for the 'low- K_m reductase' (Turner & Tipton, 1972).

The human brain hexonate dehydrogenase showed a high affinity for NADPH, with a K_m of $4.5 \mu\text{M}$ (Fig. 5), which is similar to that of the 'low- K_m ' aldehyde reductase from pig brain ($1.2 \mu\text{M}$) (Turner & Tipton, 1972). However, the value is different from that of $22 \mu\text{M}$ for the calf brain enzyme (Dons & Doughty, 1976). NADP⁺ was a competitive inhibitor of the human brain enzyme, with a K_i of $75 \mu\text{M}$ (Fig. 6). There appears to be no analogous data for NADP⁺ inhibition of the brain enzyme from other sources. For the human brain enzyme, no rate was obtained with $120 \mu\text{M}$ -NADH. Dons & Doughty

(1976) obtained a rate of 10% with NADH compared with NADPH for the calf brain enzyme.

Michaelis constants and relative maximal velocities were determined for a number of aldehyde and aldose substrates. In tissues studied to date, hexonate dehydrogenase exhibits a different substrate specificity to that of aldose reductase in that cyclized aldoses such as xylose, which are readily reduced by aldose reductase, are poor substrates for hexonate dehydrogenase. The human brain hexonate dehydrogenase showed a relatively high affinity for glucuronate (K_m of 4.6 mM) and high maximal velocity (Table 1). Although the apparent K_m for glyceraldehyde was similar to that for glucuronate, the maximal velocity with glyceraldehyde was only 50% of that with glucuronate. The cyclized aldoses xylose and glucose exhibited very low affinities (K_m values of 260 mM and 300 mM respectively) and very low maximal velocities with respect to that with glucuronate. The pattern of substrate specificity is similar to that obtained for mouse liver aldehyde reductase II (Tulsiani & Touster, 1977), which these authors concluded was identical with NADP⁺-L-gulonate dehydrogenase, i.e. hexonate dehydrogenase. Comparison with the brain hexonate dehydrogenase from other species was again restricted because of the severely limited number of common substrates used by other authors.

A number of thiol reagents (iodoacetate, iodoacetamide, *N*-ethylmaleimide and *p*-chloromercuribenzoate) were incubated with hexonate dehydrogenase to determine any inhibition by these com-

Fig. 5. Lineweaver-Burk plot for the determination of K_m value for NADPH as a substrate of human brain hexonate dehydrogenase

Assays contained 100 mM-sodium phosphate, pH 6.2, 20 mM-D-glucuronate, enzyme sample and various concentrations of NADPH in a total volume of 3 ml. Assays were carried out at 30°C and the reaction was initiated by addition of D-glucuronate. Lines were computer-fitted.

Fig. 6. Product inhibition of human brain hexonate dehydrogenase by NADP⁺ with NADPH as the variable substrate (Dixon plot)

Assays contained 100 mM-sodium phosphate, pH 6.2, 20 mM-D-glucuronate, enzyme, NADP⁺ at the indicated concentrations and one of the following NADPH concentrations: $7.5 \mu\text{M}$ (▲); $15 \mu\text{M}$ (○); $30 \mu\text{M}$ (■). Initiation of the reaction was by addition of D-glucuronate.

Table 3. *Inhibition of human brain hexonate dehydrogenase by thiol reagents*

Assays contained 100 mM-sodium phosphate, pH 6.2, 120 μ M-NADPH, enzyme and the appropriate concentrations of inhibitor. After incubation at 30°C for the indicated time, the reaction was initiated by addition of 20 mM-glucuronate. Results are expressed as a percentage of the activity of the enzyme in the absence of inhibitor.

Inhibitor	Concentration (mM)	Preincubation time (min)	Activity (%)
Iodoacetate	1.0	30	85
Iodoacetamide	1.0	30	87
	5.0	0	67
<i>N</i> -Ethylmaleimide	0.5	30	82
	1.0	30	68
	1.5	30	53
<i>p</i> -Chloromercuribenzoic acid	0.1	0	60
	0.2	0	13
2-Mercaptoethanol	5.0	0	82
	5.0	30	104
	5.0	90	165
	10.0	0	70
	10.0	30	79
	10.0	90	111

pounds (Table 3). The fact that all these reagents inhibited the brain enzyme to varying degrees suggested that maintenance of thiol groups is required for full expression of catalytic activity. Dons & Doughty (1976) found that for the calf brain enzyme 'sulphydryl reagents inhibited at all stages of purification'. For the human brain enzyme, 2-mercaptoethanol caused initial inhibition of enzyme activity but subsequent activation of activity when preincubated for up to 90 min with the enzyme. For the mouse liver aldehyde reductase II reported by Tulsiani & Touster (1977), 2-mercaptoethanol, in the same concentration range, resulted in a similar inhibition, as did 1 mM-iodoacetate. Dithiothreitol, to a final concentration of 0.2 mM, had no effect on the human brain enzyme.

The overlapping substrate specificities of aldehyde reductase, aldose reductase and hexonate dehydrogenase have given rise to varied interpretations of the enzyme species being studied. Neural aldehyde reductase is intimately associated with the metabolism of biogenic aldehydes, and hence substrate-specificity studies of this enzyme have generally employed a battery of aromatic aldehydes as substrates; and little attention has been focused on aldoses; in those cases where aldoses have been employed, the major aldehyde reductase has exhibited negligible activity with aldoses. On the other hand, studies on aldose reductase and hexonate dehydrogenase have tended to employ a battery of aldoses and aliphatic aldehydes as substrate-specificity determinants to the exclusion of aromatic aldehydes. Hence, comparisons of the aldose reductase/hexonate dehydrogenase systems with the general aldehyde reductase are difficult due to the

Table 4. *Comparison of substrate specificities of human brain hexonate dehydrogenase and Ris & von Wartburg's (1973) 'H4:3 aldehyde reductase'*

Assays on hexonate dehydrogenase were performed at 30°C and contained 100 mM-sodium phosphate, pH 6.2, 120 μ M-NADPH, enzyme sample and 0.5 mM substrate. The H4:3 enzyme was assayed at 25°C with 100 mM-sodium phosphate, pH 7.0, 160 μ M-NADPH enzyme and 0.5 mM substrate (Ris & von Wartburg, 1973). Activity is expressed as a percentage of the rate with *p*-nitrobenzaldehyde.

Substrate	Activity (%)	
	Hexonate dehydrogenase	H4:3 aldehyde reductase
<i>p</i> -Nitrobenzaldehyde	100	100
Glyceraldehyde	12.8	13.6
Benzaldehyde	4.6	6.1
4-Chlorobenzaldehyde	6.4	10.8
Pyridine-3-aldehyde	56.0	47.0
3-Nitrobenzaldehyde	10.1	10.0

specialized characterization of the enzymes on the basis of their differing functions. This absence of comparable data is most marked in the case of brain.

For the human brain, Ris & von Wartburg (1973) have demonstrated the existence of four 'multiple molecular forms' of NADPH-dependent aldehyde reductase. Large differences were found with respect to substrate specificity and inhibition by psychoactive substances for each of the four enzyme 'forms'. For substrate-specificity studies of the individual enzyme 'forms', Ris & von Wartburg

Table 5. *Inhibition specificity of human brain aldose reductase (AR) and hexonate dehydrogenase (HDH) and their comparison with the H4:2 and H4:3 aldehyde reductases described by Ris & von Wartburg (1973)*

Assays on AR and HDH were performed at 30°C with 100mM-sodium phosphate, pH 6.2, 120 μ M-NADPH, 0.5mM-*p*-nitrobenzaldehyde, enzyme sample and the inhibitor. Aldehyde reductase assays were performed at 25°C with 100mM-sodium phosphate, pH 7.0, 160 μ M-NADPH, 0.5mM-*p*-nitrobenzaldehyde, enzyme sample and inhibitor (Ris & von Wartburg, 1973).

Inhibitor	Inhibitor concentration (mM)	Inhibition (%)			
		AR	H4:2	HDH	H4:3
Barbital	1.0	22	21	91	91
Phenobarbital	1.0	45	55	81	88
Pentobarbital	1.0	26	25	85	90
Diphenylhydantoin	0.5	71	49*	93	89*
Ethosuccinimide	0.25	15	20*	20	44*

* Ris *et al.* (1975).

(1973) used a battery of substrates at one fixed concentration, namely 0.5mM for 16 of the 19 substrates tested. Similarly, each of the putative inhibitors was tested at only one concentration. To meaningfully compare the substrate specificity and inhibition characteristics of their 'multiple forms' of NADPH-dependent aldehyde reductase of human brain with the human brain aldose reductase and hexonate dehydrogenase described in the present paper, we have applied the substrate specificity and inhibitor methodology of Ris & von Wartburg (1973) to these two enzymes. Comparison of the substrate-specificity data clearly indicates that the 'H4:3 aldehyde reductase' described by Ris & von Wartburg (1973) is, in fact, hexonate dehydrogenase (Table 4). In addition, the apparent K_m of 130 μ M for *p*-nitrobenzaldehyde for 'H4:3 aldehyde reductase' (Ris *et al.*, 1975) is very similar to that of 145 μ M for hexonate dehydrogenase, as cited above. That 'H4:3 aldehyde reductase' is hexonate dehydrogenase is further confirmed by the inhibition data, which demonstrate that the extent of inhibition of hexonate dehydrogenase and 'H4:3 aldehyde reductase' by a variety of barbiturates and psychoactive drugs is identical (Table 5). These same compounds also inhibited the aldose reductase, and comparison of the inhibition of aldose reductase by these compounds with the inhibition of the 'H4:2 aldehyde reductase' species described by Ris & von Wartburg (1973) suggests that this species is, in fact, aldose reductase (Table 5). That 'H4:2 aldehyde reductase' is actually aldose reductase is further confirmed by the limited substrate-affinity data available. For 'H4:2 aldehyde reductase', the apparent K_m values of 7.3 μ M for NADPH and 9 μ M for *p*-nitrobenzaldehyde (Ris & von Wartburg, 1973; Ris *et al.*, 1975) are almost identical with the values of 3.5 μ M and 11 μ M respectively for aldose reductase as reported above. We are currently investi-

gating the nature of the inhibition of both aldose reductase and hexonate dehydrogenase by the barbiturates and other psychoactive agents.

Hence, both aldose reductase and hexonate dehydrogenase are indeed present in human brain, and under conditions of severe diabetic hyperglycaemia would thus lead to sorbitol synthesis, with the subsequent formation of fructose. This accounts for the observed presence of fructose in the cerebrospinal fluid under these conditions. Furthermore, the aldose reductase and hexonate dehydrogenase isolated from human brain and described in the present paper appear to correspond to the H4:2 and H4:3 aldehyde reductase described by Ris & von Wartburg (1973).

References

- Chrambach, A., Reisfeld, R. A., Zacchari, J. & Wyckoff, M. (1967) *Anal. Biochem.* **20**, 150–154
- Chylack, L. T., Jr. & Kinoshita, J. H. (1969) *Invest. Ophthalmol.* **8**, 401–412
- Clements, R. S., Jr., Weaver, J. P. & Winegrad, A. I. (1969) *Biochem. Biophys. Res. Commun.* **37**, 347–353
- Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
- Dons, R. F. & Doughty, C. C. (1976) *Biochim. Biophys. Acta* **452**, 1–12
- Hayman, S. & Kinoshita, J. H. (1965) *J. Biol. Chem.* **240**, 877–882
- Hayman, S., Lou, M. F., Merola, L. O. & Kinoshita, J. H. (1966) *Biochim. Biophys. Acta* **128**, 474–482
- Hers, H. G. (1957) *Le Metabolisme de Fructose*, Editions Arscia, Bruxelles
- Itzhaki, R. F. & Gill, D. M. (1964) *Anal. Biochem.* **9**, 401–410
- Jedziniak, J. A. & Kinoshita, J. H. (1971) *Invest. Ophthalmol.* **10**, 357–366
- Kinoshita, J. H., Futterman, S., Satoh, K. & Merola, L. O. (1963) *Biochim. Biophys. Acta* **74**, 340–350
- Kinoshita, J. H., Dvornik, D., Kraml, M. & Gabbay, K. H. (1968) *Biochim. Biophys. Acta* **158**, 472–475

- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mano, Y., Suzuki, K., Yamada, K. & Shimazono, N. (1961) *J. Biochem. (Tokyo)* **49**, 618–634
- Moonsammy, G. I. & Stewart, M. A. (1967) *J. Neurochem.* **14**, 1187–1193
- Ris, M. M. & von Wartburg, J. (1973) *Eur. J. Biochem.* **37**, 69–77
- Ris, M. M., Deitrich, R. A. & von Wartburg, J. (1975) *Biochem. Pharmacol.* **24**, 1865–1869
- Sheaff, C. M. & Doughty, C. C. (1976) *J. Biol. Chem.* **251**, 2696–2702
- Tulsiani, D. R. P. & Touster, O. (1977) *J. Biol. Chem.* **252**, 2545–2550
- Turner, A. J. & Tipton, K. F. (1972) *Biochem. J.* **130**, 765–772
- van Heyningen, R. (1959) *Biochem. J.* **73**, 193–207
- Varma, S. D. & Kinoshita, J. H. (1974) *Biochim. Biophys. Acta* **338**, 632–640
- Warburg, O. & Christian, W. (1941) *Biochem. Z.* **310**, 384–421