- Evans, R. J. & McGinnis, J. (1948). *J. Nutr.* **35**, 477.
 Geiger, E., Courtney, G. W. & Geiger, L. E. (1952). *Arch. Biochem. Biophys.* **41**, 74.
 Gibson, Q. H. & Wiseman, G. (1951). *Biochem. J.* **48**, 426.
 Guggenheim, K., Halevy, S. & Friedmann, N. (1960). *Arch. Biochem. Biophys.* **91**, 6.
 Gupta, J. D., Dakrouy, A. M. & Harper, A. E. (1958). *J. Nutr.* **64**, 447.
 Hagihara, H., Ogata, M., Takedatsu, N. & Suda, M. (1960). *J. Biochem., Tokyo*, **47**, 139.
 Hoeber, R. & Hoeber, J. (1937). *J. cell. comp. Physiol.* **10**, 401.
 Hou, H. C., Riesen, W. H. & Elvehjem, C. A. (1949). *Proc. Soc. exp. Biol., N.Y.*, **70**, 416.
 Jacquot, R., Matet, J. & Fridensen, O. (1947). *Ann. Nutr., Paris*, **1**, 157.
 Kamin, H. & Handler, P. (1952). *Amer. J. Physiol.* **169**, 305.
 Kratzer, F. H. (1944). *J. biol. Chem.* **153**, 237.
 Nasset, E. S. (1957). *J. Amer. med. Ass.* **164**, 172.
 Nasset, E. S., Schwartz, P. & Weiss, H. V. (1955). *J. Nutr.* **56**, 83.
 Pinsky, J. & Geiger, E. (1952). *Proc. Soc. exp. Biol., N.Y.*, **81**, 55.
 Riesen, W. H., Clandinin, D. R., Elvehjem, C. A. & Cravens, W. W. (1947). *J. biol. Chem.* **167**, 143.
 Rogers, Q. R., Chen, M. L., Peraino, C. & Harper, A. E. (1960). *J. Nutr.* **72**, 331.
 Sheffner, A., Adachi, R. & Spector, H. (1956). *J. Nutr.* **60**, 507.
 Waterlow, J. C. & Stephen, J. M. L. (1957). In *Proc. Conf Human Protein Requirements and their Fulfilment in Practice*, Princeton, U.S.A., 1955, p. 145.

Biochem. J. (1962) **83**, 135

Polyol Dehydrogenases

4. CRYSTALLIZATION OF THE L-IDITOL DEHYDROGENASE OF SHEEP LIVER*

By M. G. SMITH†

Department of Biochemistry, Medical School, University of Otago, Dunedin, New Zealand

(Received 31 August 1961)

L-Iditol dehydrogenase (sorbitol dehydrogenase) operates specifically with DPN and catalyses the reversible oxidation of several acyclic polyols to ketoses (Blakley, 1951). Fully hydroxylated pentitols, hexitols and heptitols are oxidized if they possess configuration (I) or (II) (McCorkindale & Edson, 1954), C* indicating the site of oxidation.

The enzyme occurs in the livers of all mammalian species that have been examined and procedures have been described for significant purification of the enzyme from some species (Williams-Ashman & Banks, 1954; Todd, 1954; King & Mann, 1959; Holzer & Goedde, 1960). Similar enzymic activity has been found in certain male accessory sexual glands (Williams-Ashman, Banks & Wolfson, 1957; Hers, 1957), spermatozoa (King & Mann,

1959) and guinea-pig-liver mitochondria (Hollmann & Touster, 1957). The partially purified enzyme extracted from guinea-pig-liver mitochondria (Hollmann, 1959) has been called 'DPN-xylitol (D-xylulose) dehydrogenase' (Hickman & Ashwell, 1959).

McCorkindale & Edson (1954) showed that substrates of L-iditol dehydrogenase possessing configuration (I) (sorbitol, L-iditol, xylitol) are oxidized substantially faster than those with configuration (II) (allitol, ribitol). It is possible that their rat-liver preparation contained two polyol dehydrogenases, each specific for one of these configurations, but the data of Williams-Ashman *et al.* (1957) provide evidence that a single enzyme is responsible for the oxidation of both types of polyol.

To apply a more exacting test to the unitary hypothesis, an extensive purification of the readily extractable polyol dehydrogenase of sheep liver was undertaken. A preliminary note on the crystallization of the enzyme has been published (Smith, 1960).

MATERIALS AND METHODS

Materials

Source of the enzyme. Sheep livers were obtained at the slaughter-house within 15 min. of the death of the animals and immediately chilled in crushed ice. Livers of starved

* Part 3: Shaw (1956).

† Present address: Department of Biochemistry, University of Oxford.

pregnant or lactating sheep were unsatisfactory because of their high lipid content.

Ammonium sulphate. The amount of ammonium sulphate required to reach a desired percentage saturation was calculated from the Table of Green & Hughes (1955). For the purpose of calculation all solutions were assumed to be at room temperature.

Alumina-*C*_γ gel. This was prepared by the method of Willstätter & Kraut (1923).

Organic solvents. Ethanol and acetone were distilled from commercial materials.

Polyols. Xylitol was a product of the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A., and erythritol was obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex. The sources of the other polyols have been reported earlier (McCorkindale & Edson, 1954; Shaw, 1956).

Pentuloses. D-Xylulose was prepared according to Levene & Tipson (1936) by Dr R. G. Kulka. L-Ribulose and L-xylulose were prepared from L-arabinose and L-xylose respectively by epimerization in pyridine (Glatthaar & Reichstein, 1938). As much as possible of the unchanged pentose was crystallized and removed. The syrup containing L-ribulose was treated with bromine (Hudson & Isbell, 1929) and the aldonic acids and salts were removed by ion-exchange on Amberlite IRA-400 (HCO₃⁻ form) and Amberlite IR-120 (H⁺ form). The product contained mainly L-ribulose but paper chromatography (phenol-water) revealed the presence of small amounts of aldoses and xylulose. A sample of the syrup containing L-xylulose was purified by chromatography on a column (12 cm. × 0.2 cm.²) of Dowex 1 (borate form) (Khym & Zill, 1952). Immediately after elution the fractions containing L-xylulose were mixed with Amberlite IR-120 (H⁺ form) and the boric acid was removed as volatile methyl borate (Zill, Khym & Cheniae, 1953). The product was free from aldoses, ribulose and borate.

Lactic dehydrogenase. Crystalline lactic dehydrogenase (muscle) was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.

Buffer solutions. Phosphate buffers were prepared from solutions of disodium hydrogen phosphate and potassium dihydrogen phosphate. Acetate buffers were prepared from acetic acid and sodium acetate. Glycine buffers were prepared by adjusting the pH of a solution of glycine with sodium hydroxide.

Water. Water used in the enzyme purification was distilled twice in glass apparatus.

Analytical methods

Enzyme assays. The assay of L-iditol dehydrogenase employed the DPN-linked oxidation of sorbitol to fructose. The experimental and reference cuvettes each contained 120 μmoles of sorbitol, 65 μmoles of glycine buffer, pH 9.6, and approx. 0.1 unit of enzyme, in a final volume of 2.4 ml. The reaction was started by adding 1.2 μmoles of DPN to the experimental cuvette and the extinction, *E*, at 340 mμ was read at 30 sec. intervals for 3 min. The initial velocity was determined from the graph of *E*₃₄₀ against time. The relation between enzyme concentration and initial velocity was linear. The unit of enzymic activity is defined as the amount of enzyme which reduces 1 μmole of DPN/min. at pH 9.6 and 20° in the presence of the saturating concentrations of sorbitol and DPN given above. The

specific activity of enzyme preparations is defined as the number of enzyme units/mg. of protein.

TPN-xylitol (L-xylulose) dehydrogenase was assayed by the method of Hollmann (1959), aldolase by the method of Peanasky & Lardy (1958), D-3-phosphoglyceraldehyde dehydrogenase by the method of Velick (1955) except that D-3-phosphoglyceraldehyde was formed in the cuvette by the action of aldolase on fructose 1,6-diphosphate, and alcohol dehydrogenase by the method of Theorell & Bonnichsen (1951).

Measurement of pH. This was carried out by means of a glass electrode with a Marconi Instruments pH meter.

Measurement of extinction. This was done in a Beckman DU spectrophotometer. The reaction mixtures were contained in silica cuvettes having a 1 cm. light path.

Protein estimation. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951). The standard curve was obtained with crystalline bovine plasma albumin (Armour Laboratories, Chicago, Ill., U.S.A.).

Pentulose estimation. Ribulose and xylulose were estimated by the cysteine-carbazole reaction (Dische & Borenfreund, 1951) and identified by the absorption spectrum obtained in the orcinol-ferrie chloride method (Horecker, Smyrniotis & Seegmiller, 1951).

Ultracentrifugal analysis. This was done in a Spinco model E ultracentrifuge with a 12.0 mm. standard cell. Sedimentation coefficients were determined by measuring the distance moved by the maximum ordinate of the gradient curve with a travelling microscope. The area under the curve was measured by projection of the plates and use of a planimeter. The reference base-line was obtained according to Cecil & Ogston (1948).

Purification of the enzyme

All operations were performed at 2° unless stated otherwise.

Extraction. Finely minced sheep liver (2 kg.) was mixed with 4 l. of 0.02 M-phosphate buffer, pH 7.2, and allowed to stand for 1 hr. with occasional stirring. The extract was filtered through a double layer of cheese-cloth. The specific activity of the extract was 0.03–0.04 enzyme unit/mg. of protein.

Heat treatment of the extract. Samples (2 l.) of the crude extract contained in 6 l. flasks were heated to 57° in a water bath at 65°. The extract was agitated at 57° for 8 min. and then cooled to 10° in an ice bath. The turbid solutions were combined and centrifuged at 3000g for 10 min. at 5–10°. The clear-red supernatant (specific activity 0.07–0.09 enzyme unit/mg. of protein) was kept and the copious precipitate of denatured protein discarded. About 70% of the enzyme originally in the extract was recovered.

Ammonium sulphate fractionation at 10°. Finely ground ammonium sulphate was slowly added to the mechanically stirred solution (initially at pH 7.0) until 45% saturation was attained. The solution was allowed to stand for 1 hr. before it was centrifuged at 3000g. The precipitate was discarded and the supernatant made 65% saturated with ammonium sulphate. The solution was allowed to stand for 1 hr. and then the precipitate was collected by centrifuging at 3000g, dissolved in 150–200 ml. of water and dialysed against 10 l. of water in a continuous-flow dialyser for 18 hr. at 2°. The precipitate which formed during dialysis was discarded.

Acetone fractionation. The dark-brown supernatant from the previous step was diluted to about 300 ml. with water, and 1.0M-acetate buffer, pH 5.0, was added until the solution was 0.03M with respect to acetate. The pH was then 5.2. The solution was transferred to a stainless-steel beaker standing in an ethylene glycol bath at -5° . Acetone (at -20°) was added slowly with stirring until the concentration was 27% (v/v) and the mixture was allowed to stand at -5° for 10 min. before centrifuging (1400g at -5° for 10 min.). The precipitate was discarded, the volume of the supernatant was measured, and acetone (at -20°) was added as before (except that the temperature of the solution was kept at -10°) till the concentration of acetone was 42% (v/v). The solution was allowed to stand for 10 min. at -10° , and was then centrifuged (1400g at -10° for 10 min.); the precipitate was dissolved in 0.02M-phosphate buffer, pH 6.0, to give a final volume of about 150 ml. The acetone fractionation usually increased the specific activity of the preparation two- to three-fold.

Ethanol fractionation. The dark-red solution (undialysed) from the previous step was fractionated with ethanol in a similar manner to that of the acetone treatment except that the precipitate which formed between 15 and 35% (v/v) of ethanol was collected. This precipitate was dissolved in about 100 ml. of 0.04M-phosphate buffer, pH 7.4, and dialysed against 7 l. of water for 10 hr. in a continuous-flow dialyser. The specific activity of this preparation was about 20–25 times that of the crude extract and it contained about 30% of the enzyme units originally in the liver extract.

Alumina- C_{γ} -gel fractionation. The red solution from the ethanol fractionation was adjusted to approx. pH 5.4 by the addition of a few drops of 5% (w/v) acetic acid. The solution was made 0.03M with respect to acetate buffer, pH 5.4, and the solution was then diluted with 0.03M-acetate buffer, pH 5.4, until the concentration of protein was 10 mg./ml. Sufficient alumina- C_{γ} gel was added to adsorb 20% of the enzyme. The quantity of alumina gel required for this operation was determined by a pilot experiment. The solution was allowed to stand for 15 min., centrifuged, and the gel discarded. Sufficient gel was added to the slightly coloured supernatant to adsorb the remaining enzyme, and after 15 min. the mixture was centrifuged and the supernatant discarded. The enzyme was eluted from the gel by addition of an equal volume of 0.1M-phosphate buffer, pH 7.4, and stirring until no small lumps remained. The suspension was allowed to stand for 15 min. before centrifuging. The supernatant was collected and the eluting procedure repeated thrice before the gel was discarded. This fractionation increased the specific activity of the preparation fourfold.

Second ammonium sulphate fractionation. Finely ground ammonium sulphate was added very slowly to the combined and almost colourless eluates. The solution was agitated with a magnetic stirrer and when about 30% saturation was attained it was briefly centrifuged to remove air bubbles. When sufficient ammonium sulphate had been added to make the solution 50% saturated, it was allowed to stand for 30 min., centrifuged at 10 000g and the very slight precipitate discarded. Solid ammonium sulphate was added to the supernatant until it was 62% saturated and after 1 hr. the precipitate was collected by centrifuging at the same speed. It was dissolved in the minimum volume of 0.1M-phosphate buffer, pH 7.1 (usually about 5 ml.).

Crystallization of 'aldolase'. Phosphate buffer (0.1M, pH 7.1) saturated with ammonium sulphate was added dropwise to the concentrated protein solution (20–30 mg./ml.) until a very faint turbidity appeared. This solution was kept at 2° and after about 12 hr. the sheen of the solution indicated that protein crystals were forming. At least 48 hr. was allowed for crystallization to be completed. Phase-contrast microscopy revealed masses of crystals similar in appearance to those of bovine-liver aldolase (Peanasky & Lardy, 1958). When these crystals were tested by the method of Peanasky & Lardy they were found to contain aldolase, although the specific activity was one-third that of the preparation of Peanasky & Lardy. Ultracentrifugal analysis showed at least two protein components. The crystalline precipitate with aldolase activity was removed by centrifuging at 15 000g, and an equal volume of phosphate buffer (0.1M, pH 7.1) saturated with ammonium sulphate was added to the supernatant. The precipitate was collected by centrifuging at 15 000g and dissolved in a minimal volume of 0.1M-phosphate buffer, pH 7.1 (2.3 ml.). Phosphate buffer (0.1M, pH 7.1) saturated with ammonium sulphate was added as before until a faint turbidity was observed. This solution was left for a further 12 hr. in the refrigerator and the small crop of 'aldolase' crystals removed.

The 'aldolase' crystals contained some L-iditol-dehydrogenase activity but 80–90% of the latter remained in the mother liquor, the specific activity of which was almost doubled as a result of this crystallization.

Crystallization of L-iditol dehydrogenase. The mother liquor from the second crop of 'aldolase' crystals was allowed to reach room temperature (20°) and phosphate buffer (0.1M, pH 7.1) saturated with ammonium sulphate (20°) was added dropwise until a very faint turbidity was noted. The solution was allowed to stand at room temperature and after 2–3 hr. crystals (Fig. 1) were visible under the phase-contrast microscope. After 8 hr. the crystals were collected by centrifuging. Sufficient phosphate buffer (0.1M, pH 7.1) saturated with ammonium sulphate was added to the supernatant to produce a faint turbidity and a further crop collected about 12 hr. later. A third crop could be obtained by concentrating this mother liquor as described in the previous step and repeating the dehydrogenase-crystallization procedure. Occasionally the dehydrogenase crystals (specific activity 6–10) could be seen to be contaminated with 'aldolase' crystals and even clean crystals contained some aldolase. This contaminant was removed by one or two recrystallizations.

Recrystallization of L-iditol dehydrogenase. This was accomplished by dissolving the dehydrogenase crystals in a minimum volume of 0.1M-phosphate buffer, pH 7.1, (usually less than 1 ml.) and repeating the procedure described above. When the protein concentration was sufficiently high (2–3%) and seed crystals were added, the enzyme crystallized readily, 50–60% of the units being recovered. A further crop of crystals could be obtained from the mother liquor. The specific activity of the recrystallized enzyme varied from 12.5 to 14.4. The enzyme crystals were readily soluble in water and in phosphate buffers.

Storage of the enzyme. The enzyme was precipitated from solution by adding an equal volume of phosphate buffer (0.1M, pH 7.1) saturated with ammonium sulphate, and stored at 3° . Little decrease in activity was observed after

6 months. The enzyme lost half its activity in 2 days at 20° in ammonium sulphate solution under the conditions required for crystallization.

RESULTS

The results of a typical purification of the enzyme are summarized in Table 1. The specific activity of the recrystallized enzyme is 350–400

times that of the crude extract. The yield of recrystallized dehydrogenase from 2 kg. of liver is usually less than 10 mg. This represents a recovery of about 2% of the L-iditol dehydrogenase present in the liver extract.

Absence of other enzymic activities. Recrystallized L-iditol dehydrogenase (specific activity 14.4) did not contain detectable quantities of TPN-xylitol (L-xylulose) dehydrogenase, D-3-phospho-

Fig. 1. Crystals of L-iditol dehydrogenase prepared from sheep liver (phase-contrast micrograph, $\times 160$).

Table 1. *Purification of sheep-liver L-iditol dehydrogenase*

The initial rate of oxidation of sorbitol was used for determination of activity (see Methods section).

Fraction	Volume (ml.)	Total enzyme (units)	E_{280}/E_{260}	Protein (mg./ml.)	Specific activity (units/mg. of protein)
Crude extract	3900	5300 $\pm$ 500	0.87	36.0	0.038 $\pm$ 0.003
Supernatant after heat treatment	3800	3810	0.75	12.5	0.081
Solution of fraction (dialysed) precipitated with ammonium sulphate, 45–65% saturation	250	2900	1.0	55	0.21
Solution of fraction precipitated with acetone, 27–42% (v/v)	158	2300	1.2	28	0.52
Solution of fraction precipitated with ethanol, 15–35% (v/v)	165	1520	1.4	10.8	0.85
Alumina-C ₂₅ -gel eluate	148	970	1.5	1.9	3.4
Solution of fraction precipitated with ammonium sulphate, 50–62% saturation	5.6	670	1.5	25	4.8
Supernatant after 'aldolase' crystallization	3.5	580	1.6	17	9.8
L-Iditol dehydrogenase crystals	1.2	280	2.0	21.5	10.9
Recrystallized L-iditol dehydrogenase	2.1	97	2.0	3.2	14.4

Fig. 2. Sedimentation pattern of recrystallized L-iditol dehydrogenase prepared from sheep liver. A solution of recrystallized L-iditol dehydrogenase was dialysed against a solution containing 0.01M-phosphate buffer, pH 7.3, and 0.15M-potassium chloride for 30 hr. The solution (3.2 mg. of protein/ml.) was examined in the Spinco model E ultracentrifuge in a standard 12.0 mm. cell at 20.0° and at 50 780 rev./min. Time was measured from the instant when the accelerating rotor was at 33 400 rev./min.

Fig. 3. Effect of protein concentration on the sedimentation coefficient. Recrystallized L-iditol dehydrogenase was dialysed for 24 hr. against a solution containing 0.2M-sodium chloride and 0.01M-sodium phosphate buffer, pH 7.0. Four dilutions of enzyme solution were prepared with the same buffered saline and examined in the ultracentrifuge (20.0°, 56 100 rev./min.). Sedimentation coefficients were corrected to the values in water at 20.0°.

glyceraldehyde dehydrogenase, alcohol dehydrogenase or aldolase. One recrystallization is not always sufficient to remove the last residue of aldolase. Samples of recrystallized L-iditol dehydrogenase with specific activity lower than 14.4 contained small amounts of aldolase.

Ultracentrifugal analysis. The ultracentrifuge patterns obtained with a recrystallized sample of L-iditol dehydrogenase showed a single peak (Fig. 2). Sedimentation coefficients were calculated for several concentrations of protein and $S_{20,w}^0$ was found to be 7.7 (Fig. 3). The diffusion coefficient was evaluated by the method of Hall & Ogston (1956). The graph was almost linear and gave $D_{20,w}^0$ 6.1×10^{-7} cm.²/sec. for a solution containing 3.2 mg. of protein/ml. If the partial specific volume of the enzyme is assumed to be 0.745, the molecular weight is 115 000.

Fig. 4. Effect of pH on the activity of L-iditol dehydrogenase. For sorbitol oxidation by DPN⁺ (solid line) each cuvette contained sorbitol (120 μmoles), buffer (65 μmoles) and 0.09 unit of L-iditol dehydrogenase in a final volume of 2.4 ml. The reaction was started by adding 1.2 μmoles of DPN⁺ to the reaction cuvette. For fructose reduction (broken line) each cuvette contained fructose (120 μmoles), phosphate buffer (65 μmoles) and 0.04 unit of L-iditol dehydrogenase in a final volume of 2.4 ml. The reaction was started by adding 0.27 μmole of DPNH to the reaction cuvette. Temp., 20°. ○, □, Phosphate buffer; ■, glycine buffer.

Effect of pH on velocity of reaction. L-Iditol dehydrogenase catalyses the reversible oxidation of sorbitol to fructose. The effects of pH on the rate of oxidation of sorbitol by DPN⁺ (optimum pH, 10.0) and on the rate of reduction of fructose by DPNH (optimum pH, 7.0) are shown in Fig. 4. It should be noted that whereas enzyme-saturating concentrations of sorbitol and DPN⁺ were employed, it is likely that the concentration of DPNH which had to be used for the reduction of fructose is less than that required for saturation of the enzyme.

Substrate specificity of the recrystallized enzyme. In the presence of DPN⁺, recrystallized L-iditol dehydrogenase catalysed the oxidation of xylitol (to D-xylulose), ribitol (to D-ribulose), sorbitol

(to fructose), L-itol (to sorbose), D-glycero-D-gluco-heptitol (to sedoheptulose) and L-arabitol. At pH 9.6, high concentrations (50 mM) of D-mannitol and of galactitol were oxidized at about 3% of the rate characteristic of sorbitol. Erythritol was quite inert. The ketoses mentioned above (in parentheses) are the products known to be formed (Blakley, 1951; McCorkindale & Edson, 1954).

The relative rates of oxidation of these polyols were determined by measuring the rates of reduction of DPN⁺ at pH 9.6 and 7.4 (Table 2). The concentrations of polyols (50 mM for sorbitol, ribitol and L-arabitol, 10 mM for xylitol) which gave maximum oxidation rates at both pH values were determined in preliminary experiments and used for the comparison shown in Table 2, which also includes the rates of oxidation at pH 7.4 in the presence of a physiological substrate concentration (2 mM). An additional comparison was carried out by the procedure of Hollmann (1959) who used lower substrate (6.6 mM) and coenzyme (0.13 mM) concentrations, a different pH (50 mM-tris buffer, pH 8.1) and the presence of magnesium chloride (8 mM) to study the relative rates of oxidation of polyols by the DPN-xylitol (D-xylulose) dehydrogenase of guinea-pig-liver mitochondria. The substrate concentration needed to elicit a fast rate of oxidation of xylitol is substantially less than that

required for the other polyols. This finding agrees with the observations of Hollmann (1959) on DPN-xylitol (D-xylulose) dehydrogenase. In confirmation of Hollmann's (1959) report it has been found that concentrations of xylitol exceeding 10 mM depress the rate of oxidation of this substrate. The rate of oxidation of ribitol relative to that of xylitol is greatly reduced at the lower substrate concentrations and pH values.

Michaelis constants. Michaelis constants were determined by the method of Florini & Vestling (1957) and Frieden (1957). The values at pH 9.6 were: sorbitol, 1.1 mM; ribitol, 1.8 mM; xylitol, 0.18 mM; and DPN⁺, 0.60 mM.

Evidence of enzymic homogeneity. The observation that recrystallized L-itol dehydrogenase, which exhibits a single peak in the analytical ultracentrifuge, catalyses the oxidation of polyols with both configuration (I) and (II) is evidence against the hypothesis that enzymes specific for each configuration are responsible for the polyol-dehydrogenase activity. The relative rates of oxidation of representative polyols (sorbitol, xylitol, ribitol and L-arabitol) remained constant throughout the purification procedure (Table 3).

A further test for the presence of more than one enzyme was carried out by adding two oxidizable polyols simultaneously. A mixture of sorbitol [configuration (I)] and ribitol [configuration (II)]

Table 2. *Relative rates of oxidation of polyols by recrystallized L-itol dehydrogenase*

The initial rate of reduction of DPN⁺ was measured at pH 9.6 (27 mM-glycine buffer) and at pH 7.4 (27 mM-phosphate buffer). The reactants in both reference and experimental cuvettes were contained in a final volume of 2.4 ml. and the reaction was started by addition of 1.2 μ moles of DPN⁺ to the experimental cuvette. At pH 8.1 the conditions of Hollmann (1959) were used (see text). At each pH the same amount of enzyme was used for each polyol, but owing to deterioration of activity different amounts of protein (3–8 μ g./ml.) were used in each of the four experiments. Temp. 20°. Rates of oxidation are expressed as percentages of that of xylitol. Values in the vertical columns are comparable but horizontal comparisons are not valid without reference to the initial rates of oxidation of sorbitol.

Substrate	Concn. (mM)	Relative rate of oxidation			
		pH 9.6	pH 8.1	pH 7.4	pH 7.4
Sorbitol	50.0	110 (0.095)*	—	118 (0.125)*	—
	6.6	—	75 (0.117)*	—	—
	2.0	—	—	—	59 (0.120)*
L-Iditol	6.6	—	59	—	—
Xylitol	10.0	100	—	100	—
	6.6	—	100	—	—
	2.0	—	—	—	100
Ribitol	50.0	88	—	48	—
	6.6	—	15	—	—
	2.0	—	—	—	14
D-glycero-D-gluco-Heptitol	6.6	—	30	—	—
L-Arabitol	50.0	26	—	1.0	—
	2.0	—	—	—	0.2

* Initial rate of reduction of DPN⁺ (μ moles/min.).

Table 3. *Relative rates of oxidation of polyols at stages in the purification of L-iditol dehydrogenase*

The reaction mixture contained 120 μ moles of polyol (except xylitol, of which 24 μ moles were used), 65 μ moles of glycine buffer, pH 9.6, and 0.1 unit of enzyme in a final volume of 2.4 ml. Temp., 20°. The reaction was started by adding 1.2 μ moles of DPN to the reaction cuvette. Rates of oxidation are expressed as percentages of that of sorbitol.

Fraction	Rates of oxidation			
	Sorbitol	Ribitol	Xylitol	L-Arabitol
Crude extract	100	79	89	20
Ammonium sulphate, 45–65% saturation	100	75	87	23
Ethanol, 15–35% (v/v)	100	78	88	19
Alumina-C _γ -gel eluate	100	79	90	25
Ammonium sulphate, 50–62% saturation	100	78	88	20
Recrystallized L-iditol dehydrogenase	100	79	90	23

Table 4. *Simultaneous addition of ribitol and sorbitol to L-iditol dehydrogenase*

Cuvettes contained substrate, 65 μ moles of glycine buffer, pH 9.6, and 8 μ g. of crystalline L-iditol dehydrogenase in a final volume of 2.4 ml. The reaction was started by adding 2.4 μ moles of DPN⁺ to the reaction cuvette. Temp., 20°.

Substrate	Concn. (mm)	DPNH formed (μ moles/min.)
Sorbitol	50	0.107
Ribitol	50	0.083
Sorbitol + ribitol	50 50	0.103

Table 5. *Inhibitors of L-iditol dehydrogenase*

The initial rate of reduction of DPN⁺ in the presence of sorbitol was measured at pH 9.6 under the conditions used in Table 2. Inhibitors were added to both reference and experimental cuvettes and allowed to stand for 5 min. before the addition of DPN⁺ (1.2 μ moles) to the experimental cuvette. The enzyme solution (dialysed) was prepared from the second ammonium sulphate fraction (50–62% saturation), and when necessary the inhibitor solution was adjusted to approx. pH 9.6. Temp., 20°. Initial rate of reduction without inhibitor was 0.11 μ mole of DPNH formed/min. Results are expressed as a percentage inhibition.

Inhibitor	Concn. (mm)	Inhibition (%)
<i>p</i> -Chloromercuribenzoate	0.1	100
Cysteine	2.0	50
Glutathione (reduced)	2.0	28
Potassium cyanide	1.0 10.0	5 52
<i>o</i> -Phenanthroline	0.83 0.083	50 13
EDTA	1.0	12

was oxidized a little more slowly than sorbitol alone (Table 4). Since the conditions of the experiment provided for saturation of the enzyme with coenzyme and with both substrates, an additive effect would have been expected if two enzymes were present, or if substrates with configuration (I)

reacted at a different site on the enzyme from those with configuration (II).

Inhibitors. Several compounds inhibit L-iditol dehydrogenase (Table 5). *p*-Chloromercuribenzoate (0.1 mm) completely inhibits the enzyme at pH 9.6. The other inhibitors shown in Table 5 have metal-binding properties. Cysteine (2.0 mm) and *o*-phenanthroline (0.83 mm) both inhibit the oxidation of sorbitol by 50% at pH 9.6, the degree of inhibition by cysteine being dependent on the pH. At pH 7.0 sorbitol oxidation is not inhibited by 2.0 mm-cysteine. The other listed compounds are less effective. Also, incubation of an enzyme preparation with copper sulphate (10 mm for 90 min. at pH 7.4 and 3°) causes a 80% loss of activity. These results suggest that the enzyme contains sulphhydryl groups and possibly a metal, both of which may be necessary for full activity.

Oxidation product of L-arabitol. The product of oxidation of L-arabitol has not been described by the authors who have observed the oxidation previously (Williams-Ashman & Banks, 1954; Hickman & Ashwell, 1959; Hollmann, 1959). The most likely product would be either L-xylulose or L-ribulose (Scheme 1).

Although the oxidation of L-arabitol is slow it is possible to accumulate appreciable quantities of pentulose by coupling the L-iditol-dehydrogenase system with lactic dehydrogenase. L-Arabitol (660 μ moles), sodium pyruvate (1.3 m-moles) and DPN (8 μ moles) were added to 60 ml. of glycine buffer (1.5 m-moles), pH 8.6. About 100 units of crystalline L-iditol dehydrogenase and 0.05 ml. of a slurry of lactic-dehydrogenase crystals were added to the mixture and the solution was left at room temperature. After 24 hr. 460 μ moles of pentulose had accumulated (cysteine-carbazole reaction). The solution was deproteinized with trichloroacetic acid and de-ionized by passage through a column of Amberlite IR-120 (H⁺ form) and then through a column of Amberlite IRA-400 (HCO₃⁻ form). The de-ionized solution contained a substance which gave a positive cysteine-

Fig. 5. Chromatographic analysis of the products of oxidation of L-arabitol. A column of Dowex 1 (borate form) (12 cm. $\times$ 0.2 cm.²) was prepared according to Khym & Zill (1952) and washed with 100 ml. of 5 mM-sodium tetraborate. A solution (5 ml.) of authentic xylulose (9.5 μ moles) and ribulose (6.5 μ moles) in 5 mM-sodium tetraborate was adsorbed on the column. The pentuloses were separated and eluted by 240 ml. of 0.01M-sodium tetraborate followed by 160 ml. of 0.02M-sodium tetraborate. Fractions (10 ml.) were collected at a flow rate of 1 ml./min. The temperature was 20°. A sample of each fraction was assayed for pentulose by the cysteine-carbazole reaction (Dische & Borenfreund, 1951). The peaks were identified by the orcinol-ferric chloride method (Horecker *et al.* 1951).

A sample of pentulose (about 3 mg.) formed during the oxidation of L-arabitol was made 5 mM with respect to sodium tetraborate and subjected to the same procedure. ■, Authentic xylulose and ribulose; ○, unknown pentulose.

carbazole test. Control experiments were performed by: (a) omitting L-iditol dehydrogenase from the incubation mixture; (b) incubating a purified sample of L-ribulose at pH 8.6 for 24 hr. in the absence of the enzymes. No ketose was produced in the former experiment and no xylulose was formed in the latter by non-enzymic epimerization.

The product of L-arabitol oxidation was identified as L-ribulose by chromatography on a column of Dowex 1 (borate form) (Fig. 5) and by the absorption spectrum produced in the orcinol-ferric

chloride reaction (Horecker *et al.* 1951). A small amount of xylulose was also formed.

The equilibrium of polyol-dehydrogenase reactions favours reduction of ketoses (Blakley, 1951; Hollmann & Touster, 1957; Hollmann, 1959). Since the reduction of L-xylulose to L-arabitol is a possible implication of the data of Hickman & Ashwell (1959), an attempt was made to demonstrate this reaction. Samples of L-xylulose (6 and 12 μ moles) were mixed with DPNH (0.3 μ mole) and phosphate buffer (65 μ moles), pH 7.0, final volume 2.4 ml. About 0.1 unit of L-iditol dehydrogenase was added but no oxidation of DPNH occurred.

DISCUSSION

Purification

Since there is some denaturation during crystallization at room temperature, it is not certain that the enzyme has been recrystallized to maximum or to constant specific activity. With the limits of analysis in the ultracentrifuge the enzyme appeared to be a single protein. Evidence that the purification process did not alter the relative rates of oxidation of L-arabitol, and of the polyols possessing configurations (I) or (II), is presented in Table 3. Taken with the evidence of competition between substrates (Table 4), this is a strong argument that the protein which has been crystallized is a single polyol dehydrogenase.

Substrate specificity

The rule for the substrate specificity of L-iditol dehydrogenase (Edson, 1953; McCorkindale & Edson, 1954) requires that an oxidizable polyol must have: (a) a primary carbinol group adjacent to the site of oxidation; (b) a secondary carbinol group with an L-configuration at the site of oxidation (C-2); (c) a secondary carbinol group with an L-configuration at C-4. The L-configuration is referred to the primary carbinol group at C-1.

With the exception of L-arabitol, in which the secondary carbinol group at C-4 has a D-configuration with respect to C-1, the substrates oxidized by the sheep-liver dehydrogenase (Table 2) conform to

the rule. It is probable that the enzyme will react with allitol and L-glycero-D-galacto-heptitol, which were not available but are known to be oxidized by L-iditol dehydrogenase derived from other sources.

The rate of oxidation of L-arabitol (26 % of that of xylitol) is significant only at a high pH and high substrate concentration. Since the main product of oxidation is almost certainly L-ribulose, the reaction agrees with the rule in so far as the secondary carbinol group at the site of oxidation has an L-configuration with respect to the adjacent primary carbinol group (C-1). The slow oxidation of galactitol (3 % of the rate of sorbitol oxidation) under the same conditions would be analogous if the product (undetermined) were D-tagatose. On the other hand the equally slow oxidation of D-mannitol (50 mM at pH 9.6) is contrary to the rule.

Metabolism of L-arabitol

The data of Fig. 5 show that the ketoses formed by enzymic oxidation of L-arabitol are probably about 95 % of L-ribulose and 5 % of L-xylulose. This observation, which supports the findings of Dr O. Touster (personal communication), is relevant to the possible origin of the L-arabitol that Touster & Harwell (1958) isolated from the urine from a subject with L-xyluluria. Since the failure to metabolize L-xylulose in 'essential pentosuria' is probably due to the absence of TPN-xylitol (L-xylulose) dehydrogenase in the liver, it is reasonable to suppose that some of the accumulated L-xylulose might be slowly reduced to L-arabitol by DPNH in the presence of L-iditol dehydrogenase (Touster, 1959). If the L-iditol dehydrogenase of human liver has the same specificity as that of sheep liver, this hypothesis appears to be dubious because L-xylulose was not reduced by the sheep-liver dehydrogenase under conditions in which a positive reaction could have been expected.

Comparison with DPN-xylitol (D-xylulose) dehydrogenase

Hollmann (1959) has achieved a 40-fold purification of DPN-xylitol (D-xylulose) dehydrogenase from washed mitochondria of guinea-pig liver. The complete distribution of L-iditol dehydrogenase in liver homogenates has not been determined, but there is evidence that most of this activity occurs in the fraction obtained after removal of nuclei and mitochondria (Blakley, 1951; Hollmann, 1959). Since the crystalline sheep-liver enzyme was prepared from minced liver, it could contain material derived from any part of the tissue.

There is a close similarity between the properties of Hollmann's DPN-xylitol (D-xylulose) dehydrogenase and those of L-iditol dehydrogenase obtained from sheep liver. Both enzymes are strictly

specific for DPN, and the substrate specificities are identical even in the anomalous case of L-arabitol. Hollmann (1959) has found that the mitochondrial enzyme oxidizes L-threitol and 3-deoxyxylitol (3-deoxyribitol), neither of which was available for the present work. The oxidation of both these substrates is consistent with the rule for L-iditol dehydrogenase, provided that it is assumed for L-threitol that the primary carbinol group at C-4 rotates into the same orientation as that occupied by the secondary carbinol group at C-4 of a longer polyol chain. Since 3-deoxyxylitol is oxidized faster than xylitol, Hollman has suggested that the hydroxyl substituent at C-3 of a polyol is a steric hindrance to reaction. Further, with both enzymes K_m for xylitol is considerably smaller than that for sorbitol or ribitol, and an excessive concentration of xylitol depresses the rate of oxidation of this substrate. Nevertheless, although there are significant differences between the two enzyme preparations with respect to rates of oxidation of those substrates which have been investigated under the same conditions [cf. Table 2 and Hollmann(1959)], other comparisons of kinetics are not made so easily because the experimental conditions used by different authors are different.

Differences in the kinetic properties of preparations exhibiting a specific enzymic activity are not unexpected when the protein moieties are derived either from different animal species or from different parts of the same cells. Minor differences in composition and spatial structure may affect the velocity of reaction without altering substrate specificity. Thus intra- and extra-mitochondrial DPN-linked polyol dehydrogenases might be different kinetic expressions of the same activity and may fall into the recently proposed category of 'iso-enzymes'. It is assumed in the present work that the crystalline L-iditol dehydrogenase has been extracted from the extramitochondrial portion of the cytoplasm. Some justification for this view is to be found in the observation that L-iditol dehydrogenase and glycolytic enzymes rapidly leak out of isolated hepatic cells, the mitochondria remaining intact (Berry, 1961), and in the possibility that full release of DPN-xylitol (D-xylulose) dehydrogenase from the mitochondrial residue may need treatment with butanol (Hollmann, 1959).

SUMMARY

1. L-Iditol dehydrogenase of sheep liver, which catalyses the reversible oxidation of acyclic polyols to ketoses in the presence of diphosphopyridine nucleotide, has been purified and crystallized. The specific activity of the recrystallized enzyme is 350-400 times that of the original liver extract, and about 2 % of the enzyme is recovered.

2. Ultracentrifugal analysis of the recrystallized enzyme has revealed only one protein component.

3. The optimum pH for polyol oxidation is about 10 and that for ketose reduction is about 7.

4. Michaelis constants have been determined for xylitol, sorbitol, ribitol and diphosphopyridine nucleotide. K_m for xylitol is about one-sixth of that for sorbitol and one-tenth of that for ribitol. The rates of oxidation of typical substrates have been compared under different conditions of pH and substrate concentration.

5. The substrate specificity of the enzyme conforms to the rule for L-iditol dehydrogenase except for L-arabitol; however, this anomalous oxidation occurs significantly only at high substrate concentration at a high pH.

The author wishes to acknowledge his indebtedness to Professor N. L. Edson who has given considerable help, encouragement and criticism at all stages of this work; and to thank Dr D. R. D. Shaw for many helpful suggestions.

REFERENCES

- Berry, M. N. (1961). M.D. Thesis, University of Otago.
- Blakley, R. L. (1951). *Biochem. J.* **49**, 257.
- Cecil, R. & Ogston, A. G. (1948). *Biochem. J.* **43**, 592.
- Dische, Z. & Borenfreund, E. (1951). *J. biol. Chem.* **192**, 583.
- Edson, N. L. (1953). *Rep. Austr. Ass. Adv. Sci.* **29**, 281.
- Florini, J. R. & Vestling, C. S. (1957). *Biochim. biophys. Acta*, **25**, 575.
- Frieden, C. (1957). *J. Amer. chem. Soc.* **79**, 1894.
- Glatthaar, C. & Reichstein, T. (1938). *Helv. chim. Acta*, **21**, 914.
- Green, A. A. & Hughes, W. L. (1955). In *Methods in Enzymology*, vol. 1, p. 67. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Hall, J. R. & Ogston, A. G. (1956). *Biochem. J.* **62**, 401.
- Hers, H. G. (1957). *Le Métabolisme du Fructose*, p. 134. Brussels: Éditions Arscia.
- Hickman, J. & Ashwell, G. (1959). *J. biol. Chem.* **234**, 758.
- Hollmann, S. (1959). *Hoppe-Seyl. Z.* **317**, 193.
- Hollmann, S. & Touster, O. (1957). *J. biol. Chem.* **225**, 87.
- Holzer, H. & Goedde, H. W. (1960). *Biochim. biophys. Acta*, **40**, 297.
- Horecker, B. L., Smyrniotis, P. Z. & Seegmiller, J. E. (1951). *J. biol. Chem.* **193**, 383.
- Hudson, C. S. & Isbell, H. S. (1929). *J. Amer. chem. Soc.* **51**, 2225.
- Khym, J. X. & Zill, L. P. (1952). *J. Amer. chem. Soc.* **74**, 2090.
- King, T. E. & Mann, T. (1959). *Proc. Roy. Soc. B*, **151**, 226.
- Levene, P. A. & Tipson, R. S. (1936). *J. biol. Chem.* **115**, 731.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- McCorkindale, J. & Edson, N. L. (1954). *Biochem. J.* **57**, 518.
- Peanasky, R. J. & Lardy, H. A. (1958). *J. biol. Chem.* **233**, 365.
- Shaw, D. R. D. (1956). *Biochem. J.* **64**, 394.
- Smith, M. G. (1960). *Proc. Univ. Otago med. Sch.* **38**, 6.
- Theorell, H. & Bonnichsen, R. K. (1951). *Acta chem. scand.* **5**, 1105.
- Todd, C. M. (1954). *Proc. Univ. Otago med. Sch.* **32**, 9.
- Touster, O. (1959). *Amer. J. Med.* **26**, 724.
- Touster, O. & Harwell, S. O. (1958). *J. biol. Chem.* **230**, 1031.
- Velick, S. F. (1955). In *Methods in Enzymology*, vol. 1, p. 401. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Williams-Ashman, H. G. & Banks, J. (1954). *Arch. Biochem. Biophys.* **50**, 513.
- Williams-Ashman, H. G., Banks, J. & Wolfson, S. K. (1957). *Arch. Biochem. Biophys.* **72**, 485.
- Willstätter, R. & Kraut, G. (1923). *Ber. deutsch. chem. Ges.* **56**, 1117.
- Zill, L. P., Khym, J. X. & Cheniae, G. M. (1953). *J. Amer. chem. Soc.* **75**, 1339.

Biochem. J. (1962) **83**, 144

The Relation of the Rotatory Dispersion Behaviour of Human Serum Albumin to its Configuration

BY P. CALLAGHAN AND N. H. MARTIN

Department of Chemical Pathology, St George's Hospital Medical School, London, S.W. 1

(Received 11 September 1961)

Two of the methods used for studies of the secondary and tertiary configuration of proteins in aqueous solution are deuterium-hydrogen exchange (Linderstrøm-Lang, 1958) and optical rotatory dispersion (Schellman, 1958).

There is no systematic study in the literature of the optical rotatory properties of human serum albumin and, because of variations in procedure, it is impossible to compare all the available data. In the experiments presented here we aim to demon-