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Accumulation of xylitol in the mammalian lens is related to glucuronate metabolism

Derek Goode, Mary E. Lewis, M. James C. Crabbe*

Wolfson Laboratory, School of Animal and Microbial Sciences, The University of Reading, P.O. Box 228, Whiteknights, Reading RG6 6AJ, UK

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Abstract Cataract remains the major cause of blindness worldwide and a common complication of diabetes. Polyol accumulation in the lens is associated with cataract formation. Here we present evidence for a novel pathway for xylitol production in the lens involving glucuronate metabolism. Xylitol can be produced in rat and bovine lens from glucose, via the enzymes *myo*-inositol-oxygen oxidoreductase, D-glucuronate reductase, L-gulonate NAD^+-3-oxidoreductase and L-iditol-NAD^+-5-oxidoreductase, which have been found in the mammalian lens for the first time. Glucuronate reductase has been purified and was inhibited by thiol quenching reagents. UDP-glucuronyl transferase is also present in mammalian lenses; this enzyme may be an anti-toxic defense mechanism in the lens.

Key words: Gulonate; *myo*-Inositol; Glucuronyl transferase; Xylulose; Lens; Cataract; Aging

1. Introduction

Xylose-fed young rats accumulate polyols, including xylitol [1] and develop cataractous lesions. Kinoshita and colleagues have proposed that 'sugar' cataract arises through osmotic damage to the lens as a direct result of polyol accumulation [2,3]. However, whilst xylose-fed adult rats accumulate similar polyol levels to young rats, they fail to develop cataract suggesting that the relationship between polyol levels and cataract is more complex and may involve disturbance of metabolism in rapidly growing younger lenses [4]. In order to understand how this could occur, it is necessary to elucidate the pathway by which the lens xylitol accumulates. This was originally thought to be the product of direct reduction of xylose by the lens protein AR2 (aldose reductase). However, a significant body of evidence now suggests that AR2 does not possess true enzyme activity for quantitative sugar alcohol production in the lens [5–8].

We now postulate an alternative pathway for xylitol production related to the glucuronate pathway involved in ascorbate production (Fig. 1). All of the enzymes required for this pathway have been identified in other mammalian tissues. We have now demonstrated the presence of these enzymes in rat and bovine lenses. We have studied purified glucuronate reductase, one of the key enzymes of the pathway, and identified UDP-glucuronyl transferase in rat and bovine lenses. The latter is an important toxin scavenging enzyme which has been identified in other mammalian tissues [9] but not previously reported in the lens.

*Corresponding author. Fax: (44) (01734) 318894.
E-mail: m.j.c.crabbe@rdg.ac.uk

2. Materials and methods

2.1. Preparation of lens lysates

Freshly dissected bovine or rat lenses were homgenised in 2 ml of a suitable ice-chilled assay buffer, using a manual Braun Homogeniser. Membranes and cell debris were removed by brief centrifugation in a benchtop centrifuge (at maximum speed for 2 min) and the cleared lysates were then stored on ice and assayed immediately for the enzyme of interest. If crude lysates failed to demonstrate significant enzyme activity, samples of lysate were separated by gel-filtration chromatography on a Pharmacia Hi Prep Sephacryl S-300 column and fractions in the predicted molecular weight range of the enzyme of interest were then assayed individually.

2.2. Enzyme assays

All substrates and buffer components, other than those specified separately, were obtained from Sigma Biochemicals. Spectrophotometric analysis was carried out using a Beckman DU-70 spectrophotometer with a water-heated cuvette holder.

2.2.1. myo-Inositol-1-phosphate synthetase. *myo*-Inositol-1-phosphate synthetase was assayed by a modification of the method of Eisenberg [10]. Lens lysates contain many potentially glucose-6-phosphate-utilising enzymes and addition of labeled G-6-P to a crude lens lysate would therefore yield a large number of different labeled products, making it difficult to identify any inositol-1-phosphate produced. *myo*-Inositol-phosphate synthetase has been reported in non-lens tissues as having a molecular mass of approx. 215 kDa [10]. HPLC gel filtration was used to isolate fractions of lens lysate corresponding to that molecular mass range and those fractions were then pooled and assayed for *myo*-inositol-1-phosphate synthetase activity. Incubations were carried out in 50 mM sodium phosphate buffer containing 1 mM NAD^+, 1 mM unlabeled G-6-P and 0.1 mM ^{14}C-labeled G-6-P, 208 mCi/mM obtained from Amersham (final concentrations) with and without lens lysate. Paper chromatography, using either isopropanol/water (4:1) or *n*-propanol/ethyl acetate/water (7:1:2) as the mobile phase was used to demonstrate the production of ^{14}C-labeled inositol-1-P in lens lysates incubated with ^{14}C-labeled G-6-P. Chromatograms were dried and then placed in contact with a phosphoimager screen and incubated at room temperature overnight. The phosphoimage was developed using a Molecular Dynamics Phosphoimager S1 and Image quaNT software running on a Gateway 200 4DX2-66v PC.

2.2.2. Inositol-oxygen oxidoreductase. Inositol-oxygen oxidoreductase was assayed by demonstrating an increase in glucuronate in lens lysates incubated with inositol using the orcinol method [11].

2.2.3. D-Glucuronate reductase. D-Glucuronate reductase was assayed by the method of Sivak and Hoffman-Ostenof [12].

2.2.4. L-Gulonate NAD-3-oxidoreductase. L-Gulonate NAD-3-oxidoreductase was assayed by two methods. Initial assays were performed using acetoacetate as the substrate [13]. Further assays were then carried out using L-gulonate as the substrate. L-Gulonate cannot be obtained in crystalline form as crystallisation converts it to the gulono-lactone form. It was therefore synthesised from the L-gulono-lactone using the anion-exchange resin method [14]. The L-gulonate assay was carried out in 100 mM phosphate buffer (pH 8.5), containing 1 mM NAD, 14.5 μg/ml cysteine, and approx. 40 mg/ml of synthesized gulonate. Enzyme activity was measured as a decrease in absorbance at 340 nm over 10 min at 37°C.

2.2.5. 3-Keto-L-gulonate carboxylase. 3-Keto-L-gulonate carboxylase activity was observed by demonstrating an increase in L-xylulose concentration, in lens lysates following incubation with L-gulonate. The product of L-gulonate-NAD-3-oxidoreductase acting on L-gulo-

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nate is 3-keto-L-gulonate which in the presence of active 3-keto-L-gulonate reductase gives rise to L-xylulose. Therefore, immediately after the L-gulonate assay the samples of lens lysate were deproteinized by ultrafiltration in an Amicon centrifree micropartition system. The pH was adjusted to 7.4 by addition of 100 μl of 0.5 M Tris-HCl pH 6.8 and relative concentrations of L-xylose in each lysate were determined by addition of; (final concentrations) L-xylulose reductase (0.05 U/ml), cysteine (1 mM) and NADPH (1 mM), and observing the relative rates of added L-xylulose reductase activity, determined as described in Section 2.2.6.

2.2.6. L-Iditol-NAD$^+$-oxidoreductase. L-Iditol-NAD$^+$-oxidoreductase (L-xylulose reductase) activity was assayed in crude lysates of both rat and bovine lens according to the method of Hickman and Ashwell [15].

2.2.7. UDP-glucuronyl transferase. UDP-glucuronyl transferase activity was assayed in both rat and bovine lens lysates by a modification of the method of Burchell and Weatherill [9]. Conjugation of UDP-glucuronate with 4-nitrophenol (which absorbs strongly at 405 nm) produces a colorless product. UDP-glucuronyl transferase activity was therefore measured by observing the decrease in A^{405} in lens lysates incubated for 10 min at 37°C in 0.5 M Tris-HCl, 10 mM $MgCl_2$, 0.2 mM 4-nitrophenol in the presence and absence of 20 mM UDP-glucuronate.

3. Results

Rates of reaction unless otherwise stated are in units of A^{340nm}/min.

3.1. myo-Inositol-1-phosphate synthetase

Phosphoimages of chromatograms of ^{14}C-labeled G-6-P/lens lysate incubations demonstrated the production of a single major labeled product which was not seen in the negative controls (omitting lens lysate). This product co-migrated with a control spot of unlabeled inositol-1-P (observed by developing the chromatograms with silver nitrate/sodium hydroxide reagents – data not shown) in both the isopropanol/water system in which inositol-1-P migrated ahead of G-6-P, and also in the *n*-propanol/ethyl acetate/water system in which the inositol-1-P migrated below the G-6-P (Fig. 2). This suggests that fractions of lens lysate containing proteins of 215 kDa have successfully catalysed the production of labeled inositol-1-P from G-6-P, thereby demonstrating the presence of *myo*-inositol-1-synthetase activity in the lens. On chromatography (in *n*-propanol/ethyl acetate/water) of prolonged incubations, radiolabeled spots co-migrating with authentic *myo*-inositol were observed, indicating the presence of reversible kinase activity. Similar results were obtained with rat and bovine lens lysate fractions.

3.2. Inositol-oxygen oxidoreductase

Crude rat lens lysate incubated in the presence of *myo*-inositol demonstrated a linear increase in the concentration of orcinol-positive products over time which was not observed in the absence of *myo*-inositol. With 10% bovine lens lysate the initial rates (over 30 min) were $V_{inos} = 0.0067 \pm 1.0 \times 10^{-4}$ A^{660}/min (negative control $V_{neg} = -0.0001 \pm 1.0 \times 10^{-5}$ A^{660}/min), indicating the presence of inositol-oxygen oxidoreductase in the lens. Rates of activity were proportional to concentration of lens lysates, and similar results (to within standard errors of estimation) were obtained for both rat and bovine lenses. Screening of fractions from HPLC gel filtration demonstrated the presence of this activity in fractions corresponding to molecular masses in the range of approx. 60–80 kDa which is consistent with previous reports of kidney *myo*-inositol-oxygen oxidoreductase of molecular mass 68–70 kDa [11].

3.3. D-Glucuronate reductase

An enhanced rate of NADPH depletion was observed in lens lysate incubations containing D-glucuronate compared to the negative controls (omitting glucuronate). Rates were proportional to lens lysate concentration. With 10% lens lysate $V_{gluc} = -0.012 \pm 1.0 \times 10^{-4}$ (negative control $V_{neg} = -0.004 \pm 1.0 \times 10^{-5}$). The mean enzyme activity observed in bovine lens was calculated as 2.5×10^{-3} U/mg wet weight of lens (where 1 International U = 1 μmol NADPH/min under standard conditions). Partial purification by HPLC gel filtration demonstrated similar activity in fractions corresponding to a molecular mass of 60–70 kDa. Further purification of the enzyme by subsequent ion-exchange chromatography on a DEAE-cellulose column generated a fraction with glucuronate reductase activity which on SDS-PAGE mini-gels had an apparent molecular mass of 36 kDa suggesting that the native enzyme is a dimer of two 36 kDa subunits.

3.3.1. Further characterisation of lens glucuronate reductase. Preliminary steady-state kinetic determinations on the purified lens glucuronate reductase showed classical 1:1 Michaelis Menten kinetics for both NADPH and glucuronate, with linear Eadie-Hofstee plots over concentration ranges of 200-fold and 40-fold, respectively. K_m and V_{max} values were calculated as $5.43 \pm 8.0 \times 10^{-2}$ mM and $1.2 \pm 1.0 \times 10^{-1}$ A^{340nm}/min, respectively, for D-glucuronate and as $1.20 \pm 6.0 \times 10^{-2}$ mM and $1.8 \pm 1.0 \times 10^{-1}$ A^{340nm}/min, respectively, for NADPH. Variation of assay temperature and pH demonstrated an optimal temperature of 38°C and the optimal pH as pH 7.5 with a rapid fall off in activity above pH 8.0.

The enzyme was inhibited in a time-dependent manner by the thiol quenching reagents *N*-ethylmaleimide (NEM) and *p*-chloromercuribenzoate (PCMB). Plots of log v_t/v_0 against time were linear for up to 120 min with both 15 μM NEM (slope = -5.38×10^{-3}) and 2.5 μM PCMB (slope = -5.00×10^{-3}). These results suggest the presence of an important cysteine residue situated within or close to the active site of the enzyme.

3.4. L-Gulonate NAD-3-oxidoreductase

3.4.1. Acetoacetate assay. The rate of NADH depletion in lens lysate incubations was enhanced in the presence of acetoacetate. Initial rates were proportional to concentration of lens lysate in the reaction mixture. With 10% lysate $V_{aceact} = -0.0017 \pm 1.0 \times 10^{-4}$ (negative control $V_{neg} = -0.0002 \pm 1.0 \times 10^{-5}$). The mean enzyme activity was 5.55 U/mg wet weight of bovine lens. Similar results (to within standard errors of determination) were obtained with rat lenses, showing both rat and bovine lens to contain similar levels of L-gulonate NAD-3-oxidoreductase activity. Screening of fractions from HPLC gel filtration demonstrated L-gulonate NAD-3-oxidoreductase activity in fractions corresponding to a molecular mass in the range of 60–70 kDa.

3.4.2. L-Gulonate assay. The rate of increase in A^{340} was enhanced in the presence of the synthesised L-gulonate. Initial rates were proportional to concentration of lens lysate in the reaction mixture. With 10% lens lysate $V_{gul} = 0.04 \pm 0.005$ (negative control $V_{neg} = 0.015 \pm 0.0003$); similar results were obtained in rat and bovine lenses. This confirmed the results of the acetoacetate assay demonstrating the presence of L-gulonate-NAD-3-oxidoreductase in both rat and bovine lenses.

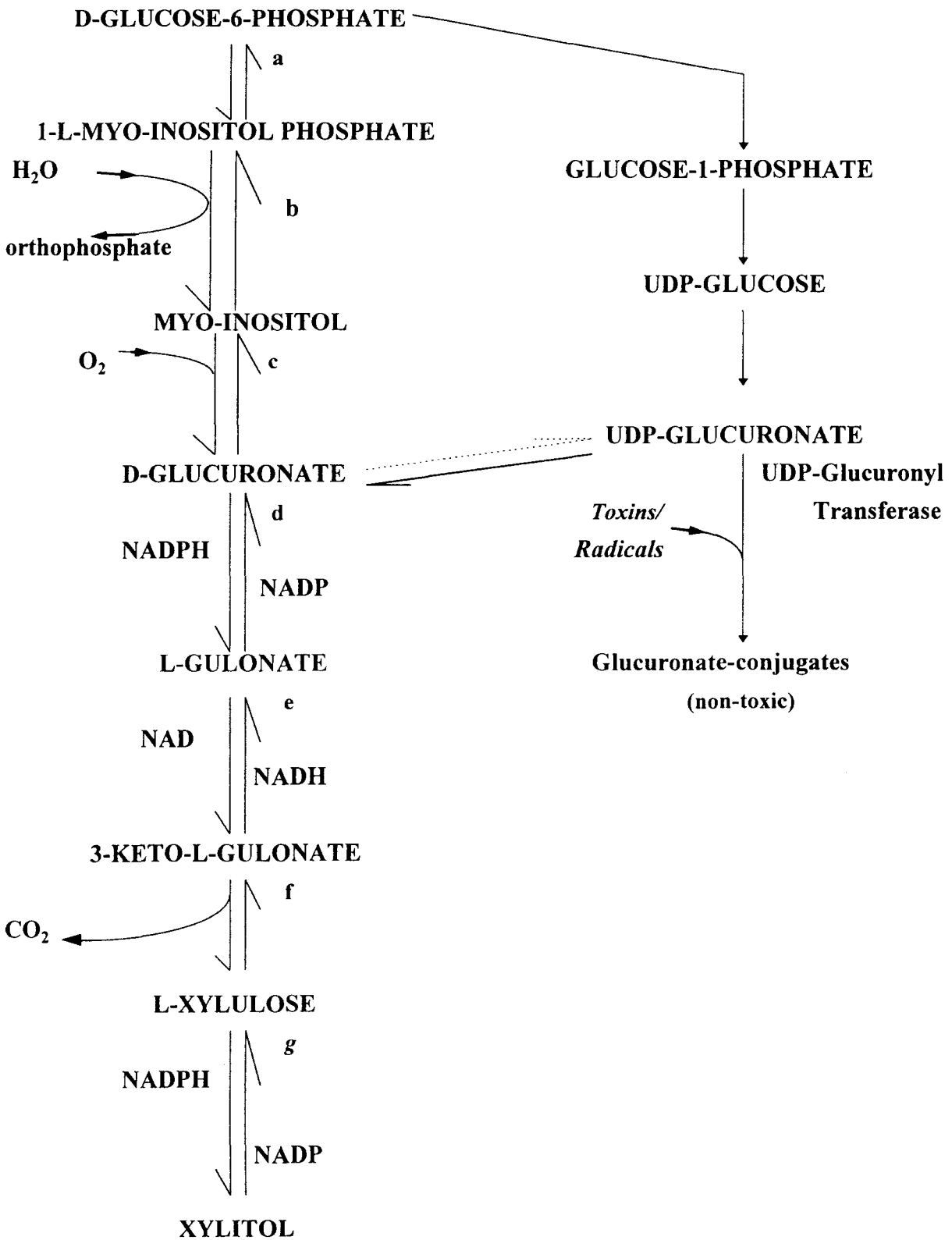

Fig. 1. Putative xylitol/glucuronate pathways in the lens. Enzymes of the xylitol pathway: a, *myo*-inositol-1-phosphate synthetase; b, 1-L-*myo*-inositol-1-phosphate kinase; c, inositol-oxygen oxidoreductase; d, D-glucuronate reductase; e, L-gulonate NAD-3-oxidoreductase; f, 3-keto-1-gulonate carboxylase; g, L-iditol-NAD^+-5-oxidoreductase.

3.5. 3-Keto-L-gulonate carboxylase

The deproteinized L-gulonate assay solutions demonstrated increased rate of added L-xylulose reductase activity in the solutions which had originally contained L-gulonate, indicating an increase in L-xylulose concentrations in those samples. Initial rates were proportional to lens lysate concentration. With 10% lens lysate $V_{xylgul} = -0.06 \pm 0.007$ (negative control

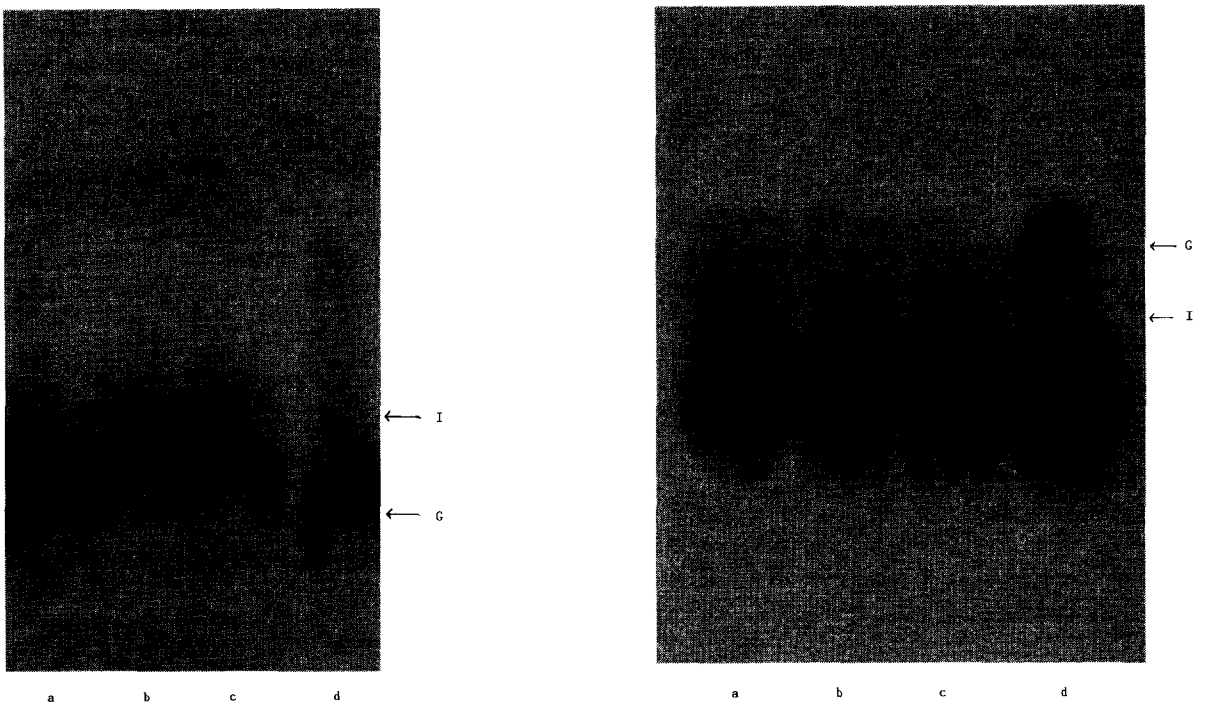

Fig. 2. Phosphoimages of paper chromatograms showing migration of ^{14}C-labelled glucose-6-phosphate derivatives produced by incubation with rat lens. (Left panel) Mobile phase=isopropanol/water; (right panel) mobile phase=*n*-propanol/ethyl acetate/water. (a–c) HPLC fractions of bovine lens lysate (142–145 ml); d, G-6-P incubated in the absence of lens protein. Arrows indicate the elution positions of G-6-P and inositol-1-P in each solvent system.

$V_{xylneg} = -0.045 \pm 0.006$). Increased xylulose reductase activity in the deproteinized lens samples, following incubation with gulonate, demonstrated increased xylulose and hence the presence of 3-keto-l-gulonate carboxylase. Similar results were obtained with rat and bovine lens lysates.

3.6. L-Xylulose reductase

Screening of HPLC gel filtration fractions succeeded in demonstrating enhanced rates of depletion of NADPH in the presence of L-xylulose in fractions corresponding to an approximate molecular mass of 60–70 kDa ($V_{xyl} = 0.013 \pm 0.001$ negative control $V_{neg} = -0.0069 \pm 0.0006$). Initial rates were proportional to protein concentration, thus demonstrating the presence of L-xylulose reductase activity in the lens. Similar results were obtained with both rat and bovine lenses.

3.7. UDP-glucuronyl transferase

Lens lysate demonstrated an enhanced rate of 4-nitrophenol depletion in the presence of UDP-glucuronate. With 10% lens lysate $V_{UDPgluc} = -0.02 \pm 0.002$ (negative control $V_{neg} = -0.0047 \pm 0.0003$). Initial rates were proportional to lens lysate concentration. Similar results were obtained for rat and bovine lenses indicating that UDP-glucuronate-transferase activity is present in the mammalian lens.

4. Discussion

The role of polyol accumulation in the mammalian lens and in cataract aetiology remains controversial. It has been suggested that far from having a deleterious effect on the lens, the accumulation of polyol may have a protective effect against high osmolarity serum [16]. The efflux of inositol from the lens fibres has been suggested as another osmoregulatory mechanism [17,18].

An understanding of inositol metabolism and the origins of polyols in the lens is required before the nature of polyol accumulation can be fully understood. We here postulate an alternative pathway for the production of xylitol in the lens, and have presented a series of simple assays which demonstrate that the enzymes required for production of xylitol via the metabolism of inositol are present in rat and bovine lenses. The xylitol pathway largely consists of the enzymes of ascorbate synthesis which have previously been demonstrated in other mammalian tissues. Man differs from rats and cattle in being unable to synthesize ascorbate but this is due to the lack of a single enzyme (gulonolactone oxidase) which is not required for xylitol production and it is therefore probable that the xylitol pathway will exist in the human lens. The flux of sugars and sugar alcohols through this pathway may be involved in the osmoregulation of the lens.

The long-lived proteins of the lens are highly vulnerable to the accumulative effects of post-translational modifications and the lens contains a number of systems to defend itself from the effects of toxic molecules, e.g. high levels of reduced glutathione. We have now demonstrated the presence in the bovine and rat lens of UDP-glucuronyl transferase activity which may represent a novel lens defence mechanism against toxic molecules such as phenols.

Cataract is a multifactorial disorder arising from a combination of factors involving post-translational modification of the crystallin proteins, decreased chaperone-like activity [19] and possibly osmotic stress. Evidence now seems to rule out AR2 as the principle enzyme of polyol metabolism in the lens. Alternative pathways involving various sugars and their deriv-

atives such as the xylitol pathway postulated here are more likely to provide a workable model of the role of polyols in sugar-cataracts.

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