Identification of Galactitol 2-Phosphate and Galactitol 3-Phosphate in the Lens of Galactose-Fed Rats

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Production of unusual phosphorylated metabolites in the lens is one of several changes caused by hyperglycemia. Sorbitol 3-phosphate (Sor-3P) and fructose 3-phosphate (Fru-3P) are two such compounds identified in the diabetic lens, and galactitol 2-phosphate (Gal-2P) and galactitol 3-phosphate (Gal-3P) are identified here in the galactosemic lens. These new compounds are the first example of galactitol metabolism in mammalian tissue other than liver. Sor-3P and Fru-3P are also present in the galactosemic lens, apparently synthesized directly from their precursors, sorbitol and fructose, which are elevated in the lens due to increased flux of glucose through the aldose reductase (AR) pathway. The NADPH necessary to support this increased flux is derived from activation of the hexose monophosphate shunt (HMPS), which is clearly demonstrated by a large increase in the concentration of sedoheptulose 7-phosphate (Sed-7P), a HMPS-specific metabolite. Additionally, during 3 weeks of galactose feeding, there is a dramatic increase in lenticular concentrations of galactitol, sorbitol, galactose, and fructose and a sharp decrease in inositol. Glucose remains unchanged. A precipitous loss of both phosphorylated and nonphosphorylated metabolites occurs after 3 weeks, possibly due to lens rupture.

CATARACT is a common cause of blindness worldwide. Senile cataract is thought to be an unavoidable and frequent accompaniment to advancing age, but sugar cataracts may appear at any age due to diabetes or other metabolic disorders. Even though a causal connection between hyperglycemia and cataractogenesis has been firmly established in diabetics, the detailed sequence of events resulting in lens opacification has not been elucidated. Among several hypotheses for the biochemical basis of sugar cataracts, osmotic stress[1-4] (elevated polyol production) and nonenzymatic glycation of crystallins[5-8] have experimental support.

Increasing the plasma level of an aldose sugar that is a substrate for the enzyme aldose reductase (AR) is one of the easiest ways to induce experimental cataracts in the rat. Under these conditions, the polyol formed can be shown to accumulate to high concentrations within the ocular lens, with the extent of accumulation being directly related to the rapidity with which cataracts develop.[9] The strongest evidence implicating the polyol pathway in sugar cataracts is that inhibition of this pathway with AR inhibitors prevents or delays cataracts in experimental models, and that animals without polyol pathway metabolism fail to develop cataracts.[10,11]

Nonenzymatic glycation of crystallins by reducing sugars is a far slower process than activation of the polyol pathway, and should slowly lead to increased protein cross-linking, sulfhydryl oxidation, and increased aggregation in crystallins from both diabetic and senile cataracts. Indeed, lens crystallins in diabetic rats are significantly more glycated than in age-matched controls.[12]

Chiou et al[13] attempted to establish a link between elevated AR activity and nonenzymatic glycation, but dismissed the possibility when the amounts of glycated protein from lenses of galactose-fed animals were unchanged regardless of whether the animal received sorbinil, an AR inhibitor. However, the identification in diabetic rat lens of fructose 3-phosphate (Fru-3P), a potent protein-glycating and cross-linking agent similar in reactivity to 3-deoxyglucose, one of the most powerful protein-glycating and cross-linking agents known, suggests that these two hypotheses are not mutually exclusive.[14]

Initial observations of ^{31}P-nuclear magnetic resonance (NMR) signals in lenses of diabetic rats, later identified by us as sorbitol 3-phosphate (Sor-3P)[15] and Fru-3P, were determined by Gonzalez et al.[16] Later, this group also observed two similar resonances in the low-field region of the ^{31}P-NMR spectrum in lenses of galactose-fed rats.[17] Neither resonance could be Sor-3P, based on their chemical shifts. However, the chemical shift of Fru-3P is close to both new resonances, raising the possibility that this potent glycating agent was elevated in this model system. Herein we eliminate this possibility by identifying these two new compounds as derivatives of galactitol, namely galactitol 2-phosphate (Gal-2P) and galactitol 3-phosphate (Gal-3P).

MATERIALS AND METHODS

Protocol

Male Sprague-Dawley albino rats (250 g) were maintained on a diet of 50% galactose (BioServ, Frenchtown, NJ). Each extract contained 10 lenses pulverized in liquid nitrogen with 5 μmol phenylphosphonic acid (an internal standard for quantitation) and 6 vol 5% perchloric acid containing 10 mmol/L trans-1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid (CDTA). Ten lenses were pooled to obtain enough material for NMR measurements. The extracts were neutralized, lyophilized to a powder, and dissolved in 25% D_2O at pH 7.5 for NMR measurements. ^{31}P-NMR spectra were obtained at 161.98 MHz on a Bruker AM 400 spectrometer (Billerica, MA) using a 10-mm probe, and were referenced to glycerophosphocholine set at 0.49 ppm. The identity of NMR was confirmed by spiking the extract with authentic compounds. Quantitation of resonances was determined by integration of peak areas, setting the phenylphosphonic acid area equal to 5 μmol. Sugars and polyols were determined on a Dionex system

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(Sunnyvale, CA) equipped with a GP40 gradient pump and an ED40 electrochemical detector using an MA1 anion-exchange column. Elution was performed with 600 mmol/L NaOH, and concentrations of metabolites were determined from standard curves of the compounds of interest. All HPLC separations included 33 μmol 2-deoxyglucose as an internal standard. Values were determined from triplicate measurements and represent the mean ± SE. Elemental analyses were performed by Galbraith Laboratories (Knoxville, TN).

Synthesis of Galactitol 2-Phosphate

Galactose 1-phosphate (50 mg, 0.2 mmol) was converted to galactose 1,2-cyclic phosphate as previously described.[18] Acid hydrolysis yielded galactose 2-phosphate as a mixture of α- and β-anomers when examined by ^{31}P-NMR (D_2O, pH 7.5, 5.00 and 5.21 ppm, phosphorous-hydrogen coupling constant [J_{POH}] 8.9 and 7.6 Hz). This was dissolved in water, and an excess of $NaBH_4$ (1 mmol) was added. After 18 hours at room temperature, the solution was neutralized with 1N HCl and chromatographed on a Sephadex G-10 column (5 × 45 cm) with water eluent. The fractions containing the target were combined and lyophilized to produce 28 mg galactitol 2-phosphate ([Gal-2P] 38% yield) with ^{31}P-NMR (D_2O, pH 7.5, 5.85 ppm, J_{POH} 10.3 Hz; analysis calculated for $C_6H_{13}O_9PNa_2 \cdot 3H_2O$: C, 20.00; H, 5.27; P, 8.61; Found: C, 19.68; H, 4.86; P, 8.46).

Synthesis of Gal-3P

For synthesis of Gal-3P,[19] 4:6-*O*-ethylidene-1:2-*O*-isopropylidene-D-galactose[20] (245 mg, 1 mmol) and diphenylphosphochloridate (300 mg, 1.2 mmol) in 10 mL pyridine were kept at room temperature for 48 hours. Pyridine was removed in vaccuo, and the residue was dissolved in toluene and filtered. The toluene solution was washed with water, 1N HCl, 1N $NaHCO_3$, and water again. Evaporation of solvent produced a thick oil that was homogeneous on silica thin-layer chromatography ($CHCl_3$:MeOH, 19:1). Phenyl groups were removed by catalytic hydrogenation over platinum oxide. Ketal groups were removed by heating an aqueous solution with Bio-Rad (Hercules, CA) AG 50W-X8 ion-exchange resin for 1 hour. The resin was filtered, and the product was purified by ion-exchange chromatography on a column (2.5 × 15 cm) of DEAE-cellulose using a gradient of 0 to 0.3 mol/L ammonium acetate, pH 5.5. The fractions containing the product were pooled, desalted on a Sephadex G-10 column, and lyophilized to produce 140 mg galactose 3-phosphate (48% yield) as a mixture of the α- and β-anomers (^{31}P-NMR, D_2O, pH 7.5, 4.59 and 4.83 ppm, J_{POH} 7.5 and 8.8 Hz). Reduction with $NaBH_4$ produced 130 mg Gal-3P (74% yield) with ^{31}P-NMR (D_2O, pH 7.5, 5.65 ppm, J_{POH} 11.7 Hz; Anal. Calcd. for $C_6H_{13}O_9PNa_2 \cdot 3.25H_2O$: C, 19.75; H, 5.35; P, 8.50; Found: C, 19.47; H, 4.92; P, 8.31).

RESULTS

Table 1 demonstrates that galactose feeding produces dramatic changes in lens concentrations of compounds associated with the polyol pathway. Galactitol increases from an undetectable concentration to 23 mmol/L within 2 days and reaches 44 mmol/L after 3 weeks of feeding. Substantial elevations in concentrations of sorbitol (0.1 to 0.4 mmol/L), galactose (0 to 1.6 mmol/L), and fructose (0.16 to 1.0 mmol/L) are also observed at 3 weeks. Inositol concentration decreases from 1.5 to less than 0.33 mmol/L, whereas glucose remains constant at 0.9 mmol/L (Fig 1). Changes in the phosphorylated compounds also occur during this period (Table 2). After 2 days, sedoheptulose

Table 1. Nonphosphorylated Metabolite Concentrations in Lenses From Galactose-Fed Rats (nmol/g wet weight of lens)

Days on Diet	Inositol	Sorbitol	Galactitol	Galactose	Fructose
0	1,475 ± 30	86 ± 6	BL	BL	162 ± 12
2	516 ± 12	131 ± 6	22,680 ± 480	70 ± 6	270 ± 12
16	96 ± 6	198 ± 6	27,120 ± 120	1,000 ± 60	510 ± 18
21	252 ± 12	275 ± 6	43,800 ± 1,800	1,100 ± 36	700 ± 18
28	20 ± 6	275 ± 12	38,580 ± 120	660 ± 60	1,000 ± 30
40	30 ± 6	6 ± 1	19,860 ± 360	55 ± 3	444 ± 48

Abbreviations: BL, below the level of detection of 2 nmol/g wet weight of lens.

7-phosphate (Sed-7P) is observed at a concentration of 0.36 mmol/L, α-glycerol phosphate (αGP) and adenosine triphosphate (ATP) are unchanged, but glycerophosphorylcholine (GPCho), glycerophosphoethanolamine (GPEt), and phosphorylcholine (PCho) decrease. More dramatic changes are apparent after 3 weeks: there is complete loss of GPCho (0.49 ppm in normal spectrum) and GPEt (1.03 ppm), αGP has increased from 0.21 to 0.45 mmol/L, and Sed-7P has reached a concentration of 3.3 mmol/L, becoming the dominant phosphomonoester resonance. PCho, which dominates the normal lens phosphorous profile, has decreased by 66% in intensity. Decreases in all phosphorylated and nonphosphorylated compounds occur after 3 weeks.

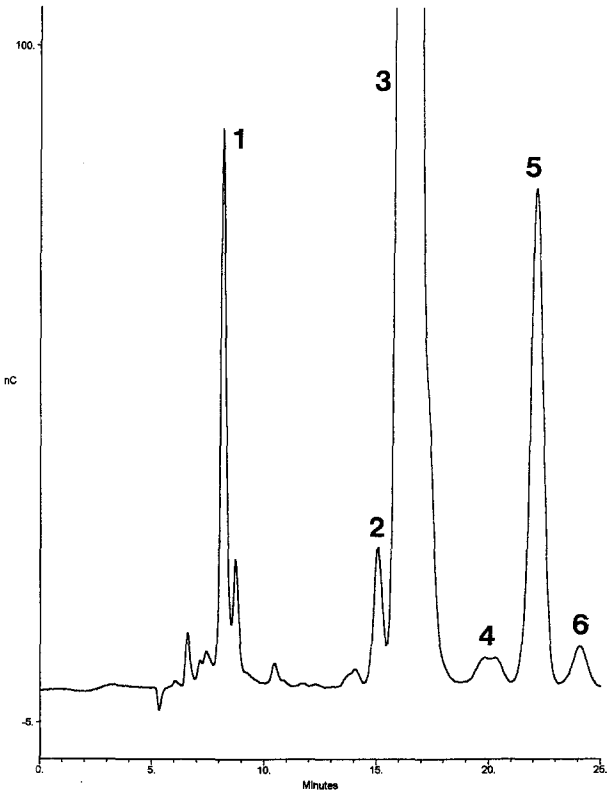

Fig 1. HPLC chromatogram of sugars and polyols from lenses of animals after 3 weeks on galactose. Individual peaks were identified and measured from standard curves: (1) inositol, (2) sorbitol, (3) galactitol, (4) glucose, (5) galactose, (6) fructose. Standards were chromatographed on the same day before and after the sample of interest to ensure peak assignment and detector response.

Table 2. Phosphorylated Metabolite Concentrations in Lenses From Galactose-Fed Rats (nmol/g wet weight of lens)

Days on Diet	Gal-2P	Gal-3P	Sed-7P	αGP	PCho	GPEt	GPCho	ATP
0	BL	BL	BL	210	6,600	285	750	2,050
2	BL	BL	360	225	5,400	240	420	2,100
21	210	280	3,300	450	2,280	BL	BL	1,600
28	150	240	2,250	270	1,420	BL	BL	930
35	150	225	1,900	300	1,320	BL	BL	960
40	90	180	1,650	270	1,260	BL	BL	900

Abbreviation: BL, below the level of detection of 10 nmol/g wet weight of lens.

Two new resonances (labeled Gal-2P and Gal-3P) are observed in the ^{31}P-NMR spectrum after 3 weeks (Table 2). Figure 2 clearly shows the new resonances at 5.65 and 5.85 ppm, which appear as doublets in the proton-coupled spectra with coupling constants of 11.8 and 10.3 Hz, respectively, and are not present in control lenses. Small resonances corresponding to Sor-3P and Fru-3P can also be observed. No change in the ^{31}P-NMR spectrum of the unknown resonances was observed when this extract was treated with sodium borohydride to reduce any carbonyl groups, demonstrating that neither compound is an aldose or ketose sugar and suggesting a polyol as the most likely precursor.

Galactose 2-phosphate and galactose 3-phosphate were synthesized and converted to Gal-2P and Gal-3P by reduction with sodium borohydride. Spiking a perchloric acid (PCA) extract with these compounds showed exact coincidence of the resonance at 5.65 with Gal-3P and that at 5.85 with Gal-2P. ^{31}P-NMR decoupled spectra of the spiked extract showed the resonances to be coincident throughout a pH titration (Fig 3).

DISCUSSION

The dramatic changes in both phosphorylated and non-phosphorylated metabolites show an active metabolism in these lenses. This is most likely due to the tremendous increase in hexose monophosphate shunt (HMPS) activity brought on by reduction of galactose to galactitol. This reduction consumes large amounts of NADPH, which activates the HMPS to provide additional amounts of this cofactor. Sed-7P, a HMPS-specific metabolite, increases from undetectable levels to 3.3 mmol/L within 3 weeks, demonstrating that the HMPS is operating at a high flux. This HMPS activation agrees with in vitro incubations of lenses in hyperglycemic medium, in which a fourfold

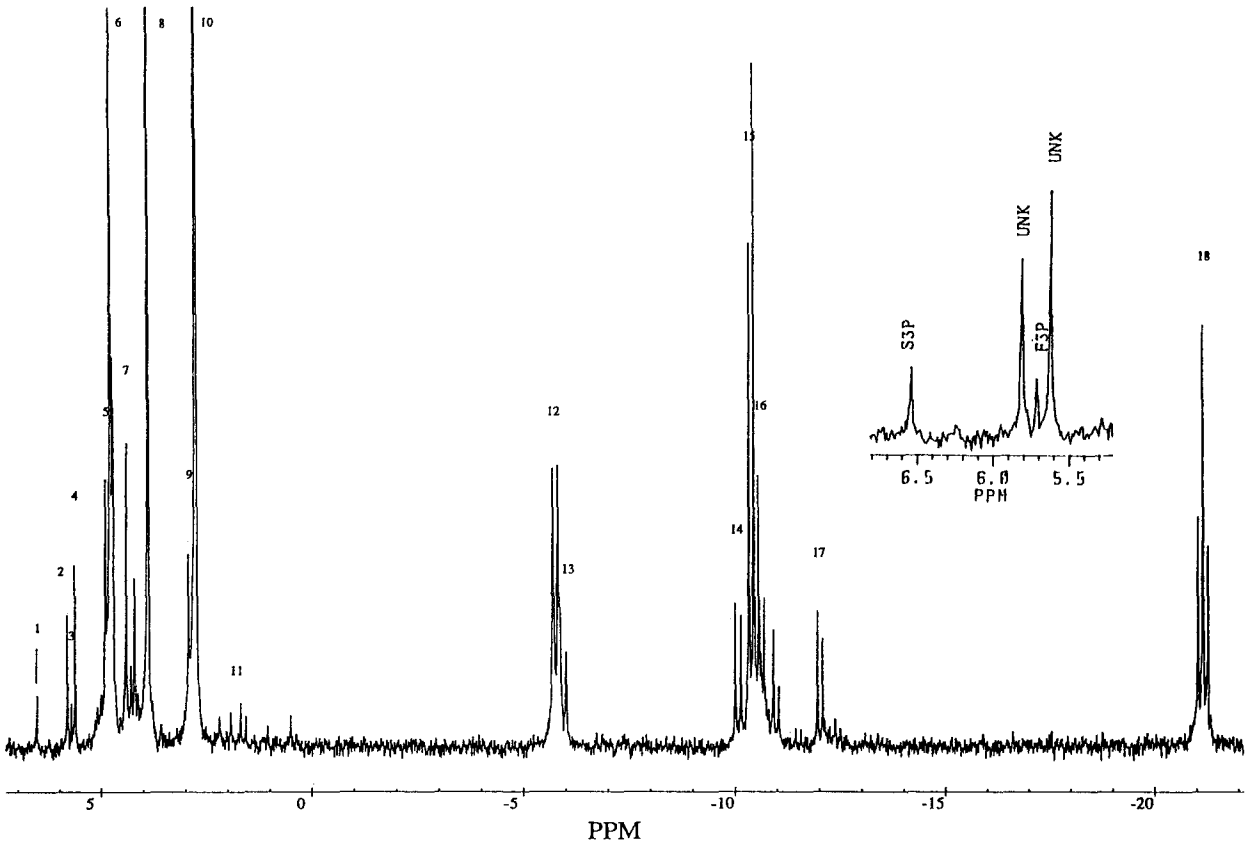

Fig 2. ^{31}P-NMR spectrum of a PCA extract of galactosemic lens. Acquisition parameters: pulse width, 6 microseconds; number of scans, 10,000; repetition time, 1.7 seconds; sweep width, 6,000 Hz. Data were transformed with a 1-Hz line-broadening filter. Peak identification: (1) Sor-3P, (2) unknown resonance at 5.85 ppm, (3) Fru-3P, (4) unknown resonance at 5.65 ppm, (5) Sed-7P, (6) αGP, (7) GPEt, (8) GPCho, (9) galactose 1-phosphate, (10) P_i, (11) inositol phosphates (12) γ-ATP, (13) β-ADP, (14) [67od] a-ADP, (15) α-ATP, (16) diphosphodiesters, (17) UDP-sugars, (18) β-ATP. Insert: An expanded view of the low-field region of the spectrum showing the unknown resonances (UNK), Sor-3P, and Fru-3P.

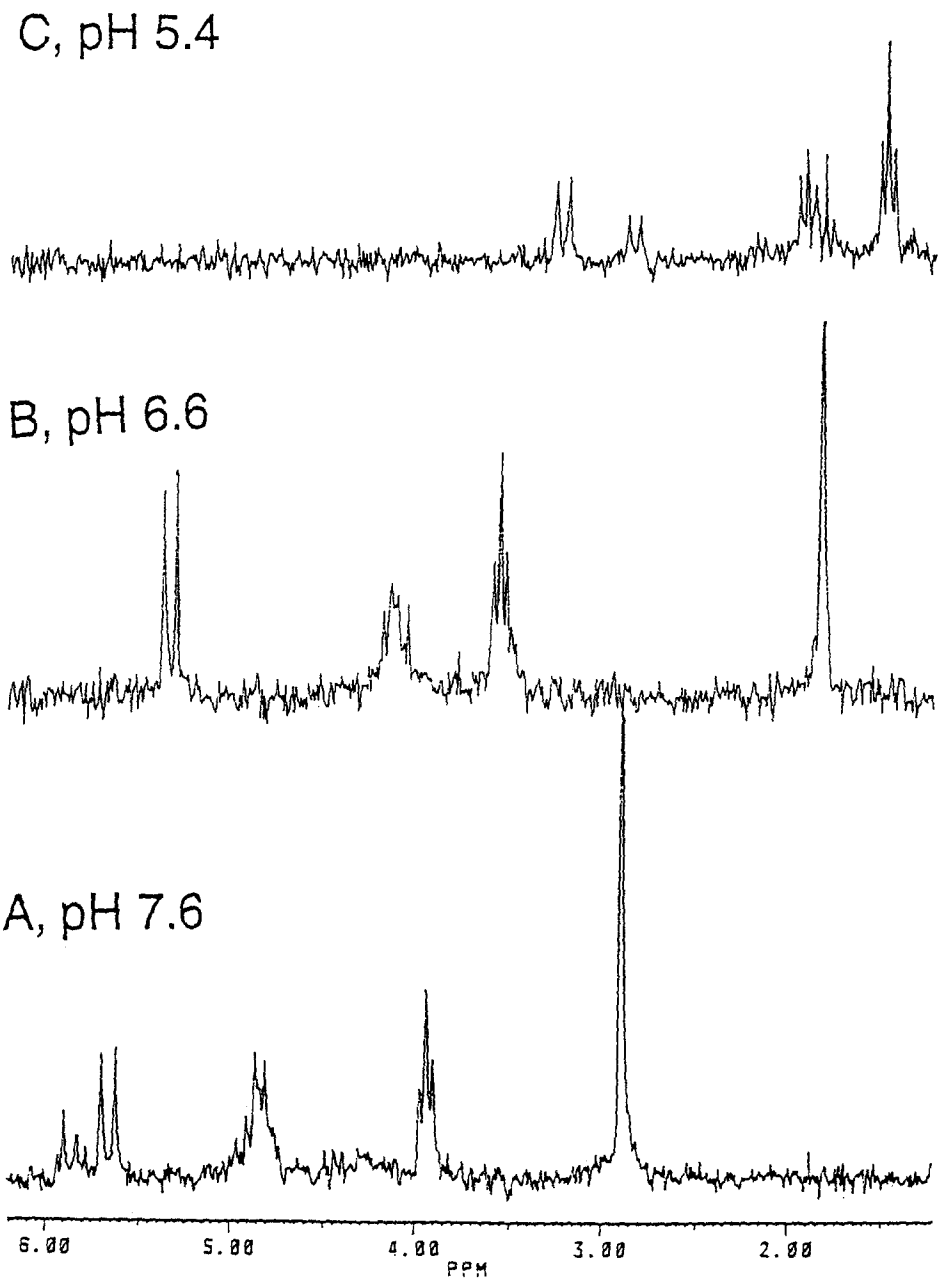

Fig 3. Coupled spectra of the low-field region of Fig 2 after the extract was spiked with Gal-3P and Gal-2P (enough to double the original peak height) acquired at (A) pH 7.6, (B) pH 6.6, and (C) pH 5.4.

elevation of glucose utilization via the HMPS was noted when rabbit lenses were incubated with 20 mmol/L galactose.[21] Also, Cheng et al[22] have shown an elevation of this pathway in rat lenses incubated with 20 mmol/L glucose. Other more subtle changes also occur in the phosphorous spectrum after 3 weeks. In addition to the appearance of two new resonances at 5.65 and 5.85 ppm, there is complete loss of GPCho and GPEt and a 66% reduction of PCho. These results are similar to those found for the diabetic lens, which also suffers complete loss of GPCho and GPEt and a 40% loss of PCho.[16] Such changes are indicative of membrane alterations, since these compounds are involved in synthesis and degradation of phospholipids.

It was obvious from Fig 2 that neither of the new resonances could be Fru-3P, because the compound is observed as a separate resonance. Therefore, galactitol phosphates became the likely candidates when the new resonances proved inert to sodium borohydride, since galactitol is present at a level of 44 mmol/L. The small amounts of the new compounds (210 nmol/g for the 5.65 resonance and 280 nmol/g for the 5.85 resonance) and the fact that only two galactitol phosphates fit NMR requirements of the phosphate ester on a secondary hydroxyl group suggested that synthesis of the most likely compounds was far more reasonable than physical isolation. Due to symmetry, Gal-2P and Gal-3P are equivalent by ^{31}P-NMR to galactitol 5-phosphate and 4-phosphate, respectively. The exact coincidence of the resonance at 5.85 ppm with Gal-2P

and the resonance at 5.65 ppm with Gal-3P during a pH titration confirms their identity. It is interesting that at near pH 5.6, Gal-3P resonates at a lower field than Gal-2P, whereas at pH 6.6 they coresonate, and at pH 7.6 Gal-2P actually resonates at the lowest field (Fig 3).[23,24] The appearance of Sor-3P and Fru-3P in galactosemic lenses was a surprise, since the animals have normal concentrations of plasma glucose (~5 mmol/L) and lenticular glucose (~1 mmol/L). Neither Sor-3P nor Fru-3P is seen in control animals, suggesting that the combined lenticular hyperglycemia of glucose and galactose (~2 mmol/L) may increase the flux of lenticular glucose through the polyol pathway, producing higher substrate concentrations of sorbitol and fructose. However, increased reduction of glucose by AR is hard to envision, since galactose is both a better substrate for the enzyme and present in a higher concentration. Alternative explanations could be activation of AR by glycation, similar to that proposed for the red blood cell,[25] or a decrease of the binding constant for glucose by either galactose or galactitol acting as an allosteric activator. Whatever the mechanism, a higher utilization of glucose via the polyol pathway in galactosemic lenses is supported by our finding of a threefold elevation in the concentration of sorbitol and a fivefold elevation of fructose in these lenses after 3 weeks of galactose feeding (Table 1). These elevated concentrations most likely explain our observation of Sor-3P and Fru-3P in these lenses.

In summary, we have identified two new phosphorylated derivatives of galactitol as Gal-2P and Gal-3P in lenses of galactose-fed rats. In addition, small amounts of Sor-3P and Fru-3P are also seen. As expected, the overwhelming change observed is the increase in galactitol concentration to 44 mmol/L after 3 weeks. The reduction of galactose to galactitol through the polyol pathway increases demand for NADPH, thereby activating the HMPS pathway, resulting in accumulation of 3.3 mmol/L Sed-7P in these lenses.

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