Direct Measurement of Polyol Pathway Activity in the Ocular Lens

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SUMMARY

A method to measure the polyol pathway metabolic flux in the intact rabbit lens by ^{13}C nuclear magnetic resonance spectroscopy is described. In the lens exposed to 35.5 mM glucose, the polyol pathway accounts for $\frac{1}{3}$ of the total glucose turnover. The high metabolic activity of the pathway suggests a significant alteration in the reduced to oxidized pyridine nucleotide ratio in the lens exposed to high glucose.
DIABETES *33*:196–199, February 1984.

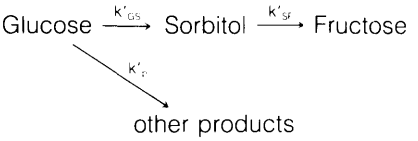
The key role played by polyol pathway activity in cataract formation in experimental diabetic models and in in vitro incubation studies is extensively documented.[1] Some of the strongest evidence implicating this pathway in "sugar" cataractogenesis comes from findings that inhibition of this pathway with aldose reductase inhibitors prevents or delays cataract formation in experimental models. In addition, animals that do not have polyol pathway enzymes will not develop cataracts when made diabetic.[1] Despite great interest in polyol pathway metabolism, the metabolic flux rate through this pathway has not been determined. We report the direct measurement of polyol pathway metabolic fluxes in the intact rabbit lens incubated under high glucose conditions as determined by ^{13}C nuclear magnetic resonance spectroscopy (NMR).

MATERIALS AND METHODS

Lenses were extracted from New Zealand White rabbits and incubated in TC-199/bicarbonate medium as previously described.[2] The incubation medium included 35.5 mM glucose enriched with ^{13}C at the C-1 position, supplied by Merck, Sharpe, and Dohme. Lenses were incubated in Petri dishes at 37°C and 100% humidity for 40–72 h. They were then transferred to NMR sample tubes for spectral analysis. The incubation medium used during spectral acquisition contained natural abundance (nonenriched) glucose. ^{13}C NMR spectra were obtained on a Bruker HX-270 spectrometer operating at 68 MHz under proton-decoupled conditions. Spectrometer parameters included a pulse width of 16 μs (≈70°), an acquisition time of 0.2048 s, a delay time of 0.3 s, 8K data points, and a sweep width of 20 KHz. An exponential filter resulting in a line broadening of 5 Hz was employed. Thirty-five hundred accumulations were obtained per spectrum. The resonance arising from the C-1 carbon of the α-anomer of glucose was used as an internal chemical shift standard.

A series of spectra of the same lens was obtained in tandem for up to 16 h without changing NMR sample tube position. Resonance peak heights were used for kinetic analysis. At the conclusion of each NMR experiment, the lens was carefully washed with balanced salt solution and frozen in liquid nitrogen. Lenticular concentrations of glucose, sorbitol, and fructose were determined enzymatically.[3,4]

Three metabolic flux rates were determined: the total glucose turnover (J_T), the rate at which glucose was metabolized to sorbitol (J_{GS}), and the rate at which sorbitol was catalyzed to fructose (J_{SF}). Standard kinetic analytic methods were employed. The following reaction model was used:



The prime superscript indicates apparent reaction constants. Thus, J_{GS} is a function of k'_{GS}, $J_{SF} = f(k'_{SF})$, and $J_T = f(k'_p) + f(k'_{GS})$.

RESULTS

It has been established that the rabbit lens incubated with 35.5 mM glucose accumulates sorbitol. After the first few hours the increase in sorbitol is linear as determined by

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Received for publication 14 November 1983.

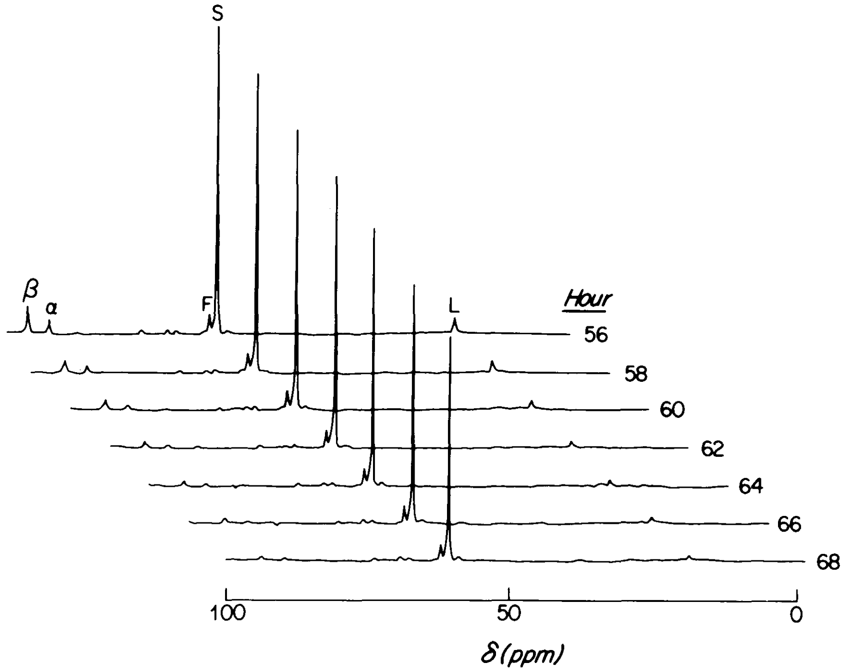

FIGURE 1. ^{13}C NMR spectra of a "pulse-chase" experiment to measure sugar metabolic fluxes in the lens. A single rabbit lens was incubated for 56 h in 35.5 mM ^{13}C-1-glucose. The medium was exchanged for one containing the same concentration of unenriched glucose, and a series of spectra were obtained in tandem for 12 h. Each spectrum was accumulated over ½ h. A partial compilation of spectra is shown here. β, α = β-, and α-anomers of glucose; F = fructose; S = sorbitol; L = lactate. Unmarked resonances derive from unenriched glucose in the medium.

biochemical assay[2] and confirmed by ^{13}C NMR.[5] We have reproduced these findings and have quantitated the rate of sorbitol accumulation (d[sorbitol]/dt) to be 0.42 μmol/g wet wt/h during 40–72 h of incubation, which is in agreement with previous reports.[2] It has also been demonstrated that the intralenticular glucose concentration does not change during this time, i.e., steady-state levels are maintained.[2,5] Using this information the total glucose turnover and the polyol pathway flux rates were determined by a "pulse-chase" experiment.[6]

Figure 1 shows a partial compilation of ^{13}C NMR spectra from a single lens that was incubated with 35.5 mM ^{13}C-1-glucose for 56 h followed by incubation with 35.5 mM natural abundance glucose. NMR spectra were obtained at ½-h intervals for the ensuing 14 h. The resonances of ^{13}C-1-glucose (both α- and β-anomers), ^{13}C-1-sorbitol, ^{13}C-1-fructose, and ^{13}C-1-lactate were identified as previously reported.[5] The glucose resonances rapidly decline in intensity and within 5 h attain "background" levels attributable to extra lenticular (medium) glucose. The sorbitol resonance increases initially, followed by a gradual decline in intensity. This is expected since sorbitol represents the second compartment of the three-compartment polyol pathway. ^{13}C-labeled fructose and lactate also show decreases in their respective resonance intensities. These changes reflect the turnover of the *labeled* metabolites, while the total lenticular concentration of sorbitol is increasing and the concentrations of glucose, fructose, and lactate are stable.

Figure 2 (top) depicts the change in the glucose resonance peak heights (α + β-anomers) as a function of time. Figure 2 (bottom) is a semilog representation of the same data for the first 5.5 h. The rate of glucose turnover (J_T) follows apparent first-order kinetics and may be described by the equation:

$$-d[^{13}\text{C-1-glucose}]/dt = k'_T[^{13}\text{C-1-glucose}]_0.$$

Integrating the above equation yields:

$$1n[^{13}\text{C-1-glucose}] = -k'_{T/2.303\ (t)} + 1n[^{13}\text{C-1-glucose}]_0.$$

Thus, the slope of the curve shown in Figure 2 (bottom) is equal to $-k'_{T/2.303}$. Because the system is at steady state the concentration of glucose at the beginning is equal to the final concentration, so that $[^{13}\text{C-1-glucose}]_0$ is equal to 10.6 μmol/g wet wt. The results are recorded in Table 1.

^{13}C-1-Sorbitol decreases linearly after the metabolism of ^{13}C-1-glucose is complete. This is expected, since total lens sorbitol is increasing reflecting saturation of the sorbitol to fructose reaction; zero order kinetics are predicted. Sorbitol turnover (J_{SF}) may then be described by the equation:

$$-d[^{13}\text{C-1-sorbitol}]/dt = k'_{SF}.$$

Integration yields:

$$k'_{SFt} = [^{13}\text{C-1-sorbitol}]_0 - [^{13}\text{C-1-sorbitol}].$$

The sorbitol terms represent the initial concentration and the concentration at time t, respectively, of the labeled sorbitol. $[^{13}\text{C-1-Sorbitol}]_0$ is estimated by the determination of the final sorbitol concentration (39.2 μmol/g wet wt) followed by extrapolation using the rate of sorbitol increase (0.42 μmol/g wet wt/h). Subsequent concentrations of the labeled metabolite are accurately determined by comparison of the resonance peak heights. k'_{SF} is equal to the slope of the curve in Figure 3 converted to concentration units. The sorbitol-to-fructose flux rate (J_{SF}) is listed in Table 1.

The rate at which glucose is converted to sorbitol (J_{GS}) is equal to the change in intralenticular sorbitol plus the rate at which sorbitol is catalyzed to fructose (J_{SF}), or:

$$J_{SF + d[sorbitol]/dt} = J_{GS}.$$

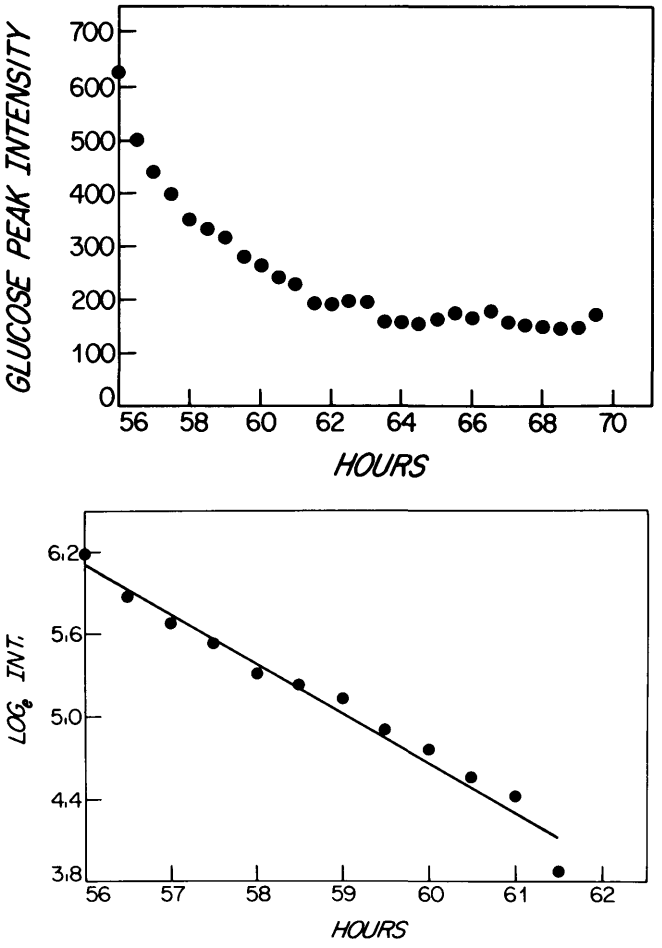

FIGURE 2. (Top) Glucose peak intensity (a sum of both the α- and β-resonances) as a function of time. (Bottom) Semilog plot of the same data for the first 5.5 h. Data derived from spectra shown in part in Figure 1.

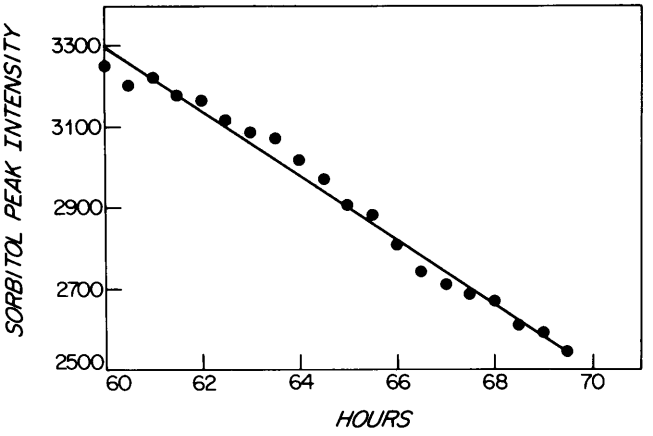

FIGURE 3. Sorbitol peak intensity as a function of time during hours 60–69.5 after lens extraction. Data derived from spectra shown in part in Figure 1.

Since both d[sorbitol]/dt and J_{SF} are linear, J_{GS} must also be essentially linear over the time period in question, and $J_{GS} = k'_{GS}$. The glucose-to-sorbitol flux rate is listed in Table 1.

Table 2 displays cofactor turnover rates attributable to polyol pathway activity using published rabbit lens cofactor concentrations.[7] The pathway flux at 35.5 mM glucose requires a greater than 3000% turnover per hour of the dinucleotide triphosphate pool and a 50% turnover per hour of the dinucleotide diphosphate pool.

The experiment described in this report was repeated four times, and the standard errors were within 10% of the values listed in Table 1. Sources of systematic uncertainty were considered. The method employed required that initial metabolite concentrations be estimated from final metabolite concentrations determined by enzyme assay. This problem is minimal for the determination of the glucose concentration since steady-state levels of this sugar are maintained during the experiment. To estimate the initial labeled sorbitol concentration, it was necessary to use an average rate of change in lenticular sorbitol (d[sorbitol]/dt) obtained from 16 lenses using an enzymatic assay. The actual rate in the experimental lens may vary somewhat from this average, but, since a large amount of sorbitol had accumulated during the incubation before the NMR experiment, even a 10% error in the estimated rate would result in only a 4% error in the true initial intralenticular concentration of ^{13}C-enriched sorbitol.

DISCUSSION

The cataractogenic role of the polyol pathway in experimental diabetic models has been attributed to the osmotic consequences of sorbitol accumulation.[1] Other possible detrimental effects have been suggested, but experimental support has been lacking. We find that the polyol pathway activity in the lens exposed to 35.5 mM glucose is surprisingly high, accounting for ⅓ of the total glucose turnover. Such high activity suggests metabolic consequences not fully appreciated previously.

Aldose reductase (AR) and polyol dehydrogenase (PD), the enzymes comprising the pathway, require the cofactors NADPH and NAD^+, respectively. High pathway activity may well result in altered reduced to oxidized pyridine nucleotide ratios. That this is the case in the lens is supported by other work from this laboratory, which demonstrated, indirectly, an

TABLE 1
Sugar metabolic flux rates in the rabbit lens

Metabolic step	Kinetic order	Flux, J (μmol/g wet wt/h)	Apparent reaction constant, k'
Glucose total*	First	3.7	0.35 h^{-1}
Glucose to sorbitol	Zero	1.2	1.2 μmol/g wet wt/h
Sorbitol to fructose	Zero	0.8	0.8 μmol/g wet wt/h

*Metabolism of glucose through all pathways.

TABLE 2
Dinucleotide cofactor turnover rates

Metabolic step	Total cofactor concentration[9]	Flux (μmol/g wet wt/h)	% Turnover per hour
$NADPH$ to $NADP^+$	0.039 μmol/g wet wt	1.2*	3000%
NAD^+ to $NADH$	1.42 μmol/g wet wt	0.8†	56%

*Aldose reductase cofactor requirement.
†Polyol dehydrogenase cofactor requirement.

elevated $NADH/NAD^+$ ratio in the lens exposed to high glucose; this effect was prevented by an AR inhibitor.[8] Altered levels of NADPH could significantly affect the health of the lens. NADPH is generated by the hexose monophosphate shunt (HMPS), and aldose reductase competes with glutathione reductase for the cofactor. The lenticular concentration of glutathione is among the highest of any organ.[9] It serves as a defense against oxidative damage and is necessary for the maintenance of certain ATP-dependent transport processes. Such a prodigious utilization of NADPH by high AR activity could explain the depression of glutathione levels found in lenses exposed to high glucose. A high polyol pathway flux and its metabolic consequences may explain the interesting finding that lenticular exposure to a combination of high glucose and oxidative stress results in greater damage to the lens (as reflected by cation transport and morphology) than either stress alone, despite lower levels of sorbitol accumulation.[10]

The sensitivity of NMR is such that we were unable to determine the polyol pathway flux in the lens exposed to 5 mM glucose, because intralenticular glucose and sorbitol are not detected at that concentration. The question arises whether polyol pathway fluxes are different under high and normal glucose exposure. Studies of lens HMP shunt activity suggest that they are in fact different. The HMP shunt is the only significant source of NADPH in the lens, and NADPH is required by AR. Giblin et al.[7] reported that the rabbit lens exposed to 5 mM glucose produces 0.19 μmol/g wet wt/h of NADPH by HMPS activity. Kinoshita et al.[11] reported similar findings as well as a fivefold increase in HMPS activity in the lens exposed to 30 mM glucose. Thus, it would seem that the polyol pathway flux is reflected by HMPS activity and is at least some five times greater at 35.5 mM than at 5 mM glucose.

The present study demonstrates a method employing ^{13}C NMR by which polyol pathway fluxes may be determined. Exposure of the lens to high glucose results in high pathway activity accounting for $\frac{1}{3}$ of the total glucose turnover. Further studies employing different glucose concentrations as well as continuously varying sugar concentrations should provide additional information on the dynamics of the polyol pathway.

ACKNOWLEDGMENTS

We gratefully acknowledge Dr. Leo Neuringer and the Francis Bitter National Magnet Laboratory at the Massachusetts Institute of Technology for the use of their facility under grant #RR00995 from the Division of Research Resources of the NIH, and by the National Science Foundation under contract #C-670.

This project was supported by research grants EY 01276, EY 04424, and EY 07063 from the National Eye Institute.

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