

Sources of hepatic glycogen synthesis in mice fed with glucose or fructose as the sole dietary carbohydrate

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Purpose: The positional analysis of hepatic glycogen enrichment from deuterated water (²H₂O) by ²H NMR has been applied previously to resolve the contributions of glucose and fructose to glycogen synthesis in rodents fed a high sucrose diet. To further validate this method, this analysis was applied to mice fed with synthetic diets whose carbohydrate components consisted solely of either glucose or fructose.

Methods: Eight glucose-fed and 12 fructose-fed mice were given ²H₂O followed by ad libitum feeding overnight. Mice were then euthanized, hepatic glycogen was isolated and derivatized to monoacetone glucose, and ²H-enrichment of positions 2, 5, and 6_S were measured by ²H NMR. From these data, the fraction of overnight glycogen appearance from the direct pathway and/or glycogen cycling and indirect pathway were estimated. Indirect pathway fractions were resolved into Krebs cycle and triose-phosphate sources—the latter including contributions from fructose metabolism.

Results: After overnight feeding, the fraction of overnight glycogen appearance derived from direct pathway and/or glycogen cycling in glucose-fed-mice was 63 ± 1%. For the indirect pathway, Krebs cycle and triose-phosphate sources contributed 22 ± 1% and 15 ± 1%, respectively. For fructose-fed-mice, glycogen appearance was dominated by triose-phosphate sources (60 ± 2%) with lesser contributions from Krebs cycle (14 ± 1%) and direct and/or glycogen cycling (26 ± 2%).

Conclusions: ²H NMR analysis of hepatic glycogen ²H enrichment from ²H₂O provides realistic profiles of dietary glucose and fructose contributions to hepatic glycogen synthesis in mice fed with diets containing 1 or the other sugar as the sole carbohydrate source.

KEYWORDS

deuterated water, gluconeogenesis, ²H NMR, indirect pathway

1 | INTRODUCTION

In Westernized societies, the surge in obesity and related complications such as type 2 diabetes and nonalcoholic fatty liver disease has in part been attributed to increased

consumption of refined sugar in the form of sucrose or high-fructose corn syrup. Because the liver is among the first sites in the body to intercept glucose and fructose from these sources, there is substantial interest in hepatic metabolism of these sugars. In contrast to glucose, hepatic conversion of

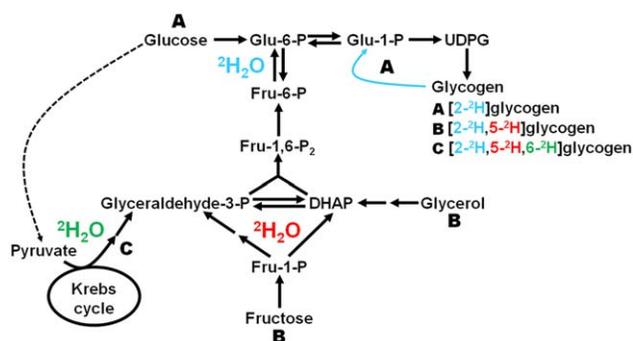


FIGURE 1 Schematic of glycogen synthesis from fructose and glucose and positional enrichment of glycogen from $^2\text{H}_2\text{O}$. For clarity, some metabolic intermediates have been omitted. A, Glycogen derived through direct pathway metabolism of glucose (Glucose \rightarrow Glu-6-P \rightarrow Glu-1-P \rightarrow Uridine diphosphate glucose (UDPG) \rightarrow Glycogen) or undergoing cycling following hydrolysis to Glu-1-P (blue arrow) will be enriched in position 2. B, Glycogen derived from triose-P sources, including fructose, will be enriched in positions 2 and 5. C, Glycogen derived from pyruvate (which may originate from glucose metabolized peripherally through the Cori cycle or from hepatic glycolysis) or from any other anaplerotic Krebs cycle substrate (not shown) will be enriched in positions 2, 5, and 6_S

fructose to triose phosphates (triose-P) is not regulated by insulin. When dietary fructose is abundant, the resulting high inflow of triose-P promotes hepatic glycogen synthesis through the indirect pathway (Figure 1). We previously demonstrated in rat models that glycogen synthesis through direct and indirect pathways can be quantified during natural overnight feeding using deuterated water ($^2\text{H}_2\text{O}$).¹ Subsequently, we demonstrated that indirect pathway contributions can be additionally resolved into substrates that enter at the level of triose-P, which include fructose, and those that are metabolized through Krebs cycle anaplerosis.² This analysis revealed a significant increase in indirect pathway triose-P contributions to glycogen synthesis in rats whose normal chow diet was supplemented with sucrose in the drinking water. This increase in triose-P contribution was explained by glycolytic metabolism of the fructose component of sucrose. Because dietary glucose sources were more plentiful than that of fructose in these studies (i.e., glucose equivalents from the drinking water sucrose plus additional glucose derived from maltose in the chow),¹ we hypothesized that if fructose was the dominant dietary sugar, then hepatic glycogen synthesis should be skewed even further towards indirect pathway triose-P sources and this would be reflected in the ^2H -enrichment distribution of glycogen. Conversely, if fructose was completely absent and dietary carbohydrate consisted entirely of glucose, then hepatic glycogen synthesis from triose-P sources should be minimal, whereas direct pathway contributions would dominate. We tested this hypothesis by measuring the sources of hepatic glycogen synthesis in 2 groups of mice: 1 group fed a synthetic diet in

which fructose was the sole carbohydrate component and the other fed a diet with glucose as the sole carbohydrate.

2 | METHODS

2.1 | Materials

The $^2\text{H}_2\text{O}$ at 99.9% enrichment was obtained from Eurisotop (Saint-Aubain, France).

2.2 | Animal studies

Animal studies were approved by the University of Coimbra Ethics Committee on Animal Studies and the Portuguese National Authority for Animal Health (approval code 0421/000/000/2013). Adult male C57BL/6 mice were obtained from Charles River Labs (Barcelona, Spain) and housed at the University of Coimbra Faculty of Medicine Bioterium, where they were maintained with a 12-hour light/12-hour dark cycle. Following delivery to the Bioterium, mice were provided a 2-week interval for acclimation, with free access to water and standard chow, consisting of 60% mixed carbohydrate, 16% protein, and 3% lipid. After this period, animals were randomly assigned to 2 synthetic diets formulated on an AIN-93G background and supplied by Special Diets Services (Argenteuil, France) for a 10-week period. The first formulation consisted of 60% glucose, 16% protein, and 3% lipid by weight and the second consisted of 60% fructose, 16% protein, and 3% lipid by weight. These synthetic diets were packaged in coarse powder form. For this reason, the mice were provided with the powdered standard chow placed in small open Petri dishes during the initial 2-week adjustment period, and this method of feed delivery was used in the subsequent studies. Fasting glucose levels and glucose tolerance were assessed at baseline and at the start of weeks 5 and 10 of the feeding trial. Mice were fasted throughout the dark period and through the initial 4 hours of the light period for a total time of 16 hours. They were gavaged with a solution of 10% glucose prepared in sterilized drinking water whose volume corresponded to 2 mg glucose/g body weight. Blood glucose levels were monitored from tail tip samples at 0, 15, 30, 60, and 120 minutes after gavage using a OneTouch Vita (LifeScan, Milpitas, CA) glucometer, and glucose tolerance was assessed by the area under the curve over 0 to 120 minutes.

On the final evening of the feeding trial, mice were given an intraperitoneal injection of 99.9% $^2\text{H}_2\text{O}$ with 0.9% weight/volume NaCl at a dose of 3 g/100 g body weight at the start of the dark cycle. At the same time, the drinking water was supplemented with 99.9% $^2\text{H}_2\text{O}$ (5% volume/volume). At the end of this dark cycle, mice were anesthetized with halothane and sacrificed by cervical dislocation. Arterial blood was collected and rapidly centrifuged for plasma

collection and livers were freeze-clamped. Plasma and livers were stored at -80°C until further analysis. In a separate study, 12 adult mice fed with standard chow were maintained under the same conditions. Their drinking water was supplemented at the start of the dark period with 15% weight/weight glucose enriched to 20% with $[\text{U-}^2\text{H}_7]\text{glucose}$, and 15% weight/volume unlabeled fructose. After ad libitum feeding and drinking overnight, the mice were euthanized, and liver glycogen was extracted and analyzed by ^2H NMR as described subsequently. The ratio of glycogen enrichment in positions 2 and 3 was used to calculate the correction factor for incomplete glucose-6-phosphate (G6P)–fructose-6-phosphate exchange as previously described.⁶

2.3 | Glycogen extraction and monoacetone glucose synthesis

Samples were prepared as described previously by Rito et al.³ Briefly, glycogen was extracted from frozen liver powder by treatment with 30% potassium hydroxide (2 mL/g of liver) at 70°C for 30 minutes. The mixture was treated with 6% Na_2SO_4 (1 mL/g of liver) and glycogen precipitated with ethanol (7 mL/g of liver). After centrifugation, the solid residue was dried and resuspended in acetate buffer (0.05 M, $\text{pH} = 4.5$). Aqueous solution containing 16 U of amyloglucosidase from *Aspergillus niger* (Glucose-free preparation, Sigma-Aldrich, Germany) was added and incubated overnight at 55°C . The supernatant was lyophilized and mixed with 5 mL ^2H -enriched acetone prepared as described⁴ and 4% sulfuric acid enriched to 2% with $^2\text{H}_2\text{SO}_4$ (volume/volume). The mixture was stirred overnight at room temperature. The reaction was quenched with water (5 mL, enriched to 2% with $^2\text{H}_2\text{O}$), the pH adjusted with HCl ($\text{pH} 2.0$), and the mixture incubated at 40°C for 5 hours. The solution pH was adjusted to 8 with NaHCO_3 and the samples evaporated to dryness. Monoacetone glucose in the residue was extracted with boiling ethyl acetate. Ethyl acetate was evaporated, the residue dissolved in H_2O and purified by solid-phase Discovery DSC-18 3-mL/500 mg disposable columns (Sigma-Aldrich) as previously described.³

2.4 | Nuclear magnetic resonance analysis

Proton-decoupled ^2H NMR spectra were obtained with a Varian VNMRs 600 MHz NMR (Agilent, Santa Clara, CA) spectrometer equipped with a 3-mm broadband probe. Plasma body water ^2H -enrichments were determined from 10 μL of plasma by ^2H NMR as described previously.⁵ Monoacetone glucose samples were dissolved in 90% acetonitrile/10% water and the ^2H NMR spectra were acquired at 50°C using a 90° pulse and 1.7 seconds of recycling time (1.6 seconds of acquisition time and 0.1-second pulse delay). The spectra were processed with 1.0-Hz line-broadening

before Fourier transformation. Positional ^2H enrichments were determined from the known enrichment of monoacetone glucose methyl signals that were used as an internal standard. Spectra were analyzed using NUTS PC-based NMR spectral analysis software (Acorn NMR Inc, Livermore, CA).

2.5 | Quantification of the sources of hepatic glycogen appearance

The fractional contributions of direct pathway, indirect pathway sources using the Krebs cycle and indirect pathway sources using triose-P to overnight glycogen appearance were quantified from glycogen positions 2, 5, and 6_S (H2, H5, and H6_S) using the following 3 equations²:

$$\text{Direct pathway and/or glycogen cycling} = 100 \times (1 - \text{H5}/\text{H2}) \quad (1)$$

$$\text{Indirect pathway sources using Krebs cycle} = 100 \times \text{H6}_S/\text{H2} \quad (2)$$

$$\text{Indirect pathway sources using triose-P} = 100 \times (\text{H5} - \text{H6}_S)/\text{H2} \quad (3)$$

H2 was adjusted for the known incomplete exchange of body water and position 2 hydrogens through G6P isomerase.⁶ Because glycogen cycling (glycogen \rightarrow Glu-1-P \leftrightarrow Glu-6-P \leftrightarrow Fru-6-P \leftrightarrow Glu-6-P \leftrightarrow Glu-1-P \rightarrow UDPG \rightarrow glycogen) can result in the selective enrichment of position 2,⁷ thereby mimicking direct pathway contributions, we reported this activity as direct pathway and/or glycogen cycling.

2.6 | Statistics

All results are presented as means \pm standard error and comparisons were made by an unpaired t-test (2-tailed) performed using Microsoft Excel.

3 | RESULTS

The 2 groups of mice showed equal weight gains over the 10-week feeding trial (24.0 ± 0.4 g to 26.2 ± 0.6 g for glucose-fed mice (GLU) and 22.6 ± 0.6 g to 24.6 ± 0.4 g for fructose-fed mice (FRU)). The 2 groups also had equal fasting glucose levels and glucose tolerance at baseline as well as in the 5th and 10th week of feeding (data not shown). Body water enrichments had a tendency to be higher in FRU mice compared with GLU mice (Table 1), but the difference was not significant ($P = 0.18$). Figure 2 shows representative ^2H NMR spectra of derivatized glycogen obtained from a FRU mouse and a GLU mouse. The spectra had well-resolved signals with high SNRs from all 7 hydrogens

TABLE 1 Glycogen and body water ^2H -enrichments and fractional contributions of various sources to overnight glycogen appearance following $^2\text{H}_2\text{O}$ administration for mice fed with diets in which the carbohydrate component consisted either entirely of fructose or of glucose

| Diet | Glycogen positional enrichments and body water enrichment | | | | | | | Glycogen appearance sources (%) | | | | |
|------------------------------|---|-------------|----------------|-------------|-------------|-------------|-------------|---------------------------------|-------------|-----------------------|---------------------|----------------------|
| | 1 | 2 | 2 ^a | 3 | 4 | 5 | 6R | 6S | Body water | Direct and/or cycling | Indirect triose-P | Indirect Krebs cycle |
| Fructose (<i>N</i> = 12) | 0.67 (0.09) | 1.43 (0.04) | 2.26 (0.07) | 1.32 (0.07) | 1.52 (0.04) | 1.66 (0.05) | 0.35 (0.02) | 0.32 (0.01) | 2.70 (0.01) | 26 ^b (2) | 60 ^c (2) | 14 ^d (1) |
| Glucose (<i>N</i> = 8) | 0.59 (0.09) | 1.01 (0.14) | 1.60 (0.23) | 0.46 (0.07) | 0.58 (0.08) | 0.59 (0.09) | 0.36 (0.06) | 0.38 (0.07) | 2.26 (0.07) | 63 (1) | 15 (1) | 22 (1) |

Note: Data are shown as means with standard errors in parentheses below each value.

^aPosition 2 enrichment after correction for incomplete exchange of ^2H between water and glucose-6-phosphate position 2.

^b $P = 3 \times 10^{-10}$ compared with glucose.

^c $P = 1 \times 10^{-12}$ compared with glucose.

^d $P = 2 \times 10^{-4}$ compared with glucose.

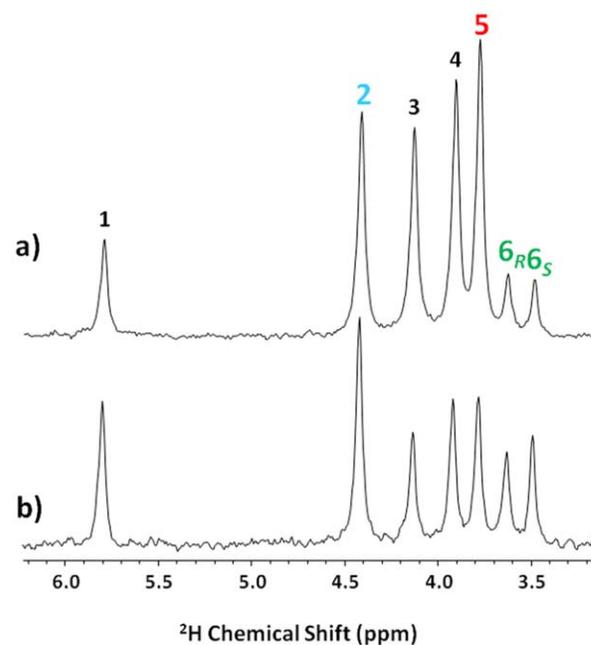


FIGURE 2 ^2H NMR spectra of monoacetone glucose derived from liver glycogen of a mouse fed a diet in which fructose was the sole carbohydrate (a) and a diet in which glucose was the sole carbohydrate (b). The numbers above the signals indicate their glycosyl positional origin

attached to the hexose carbon skeleton, allowing precise quantification of positional ^2H enrichment in all sites (Table 1). The glycogen spectrum from the FRU mouse showed low enrichment of position 6_S (as well as 6_R) relative to position 5, indicating a high abundance of glycogen enriched in position 5 but not in position 6_S over that enriched in both positions. As indicated by Figure 1, this implies a high contribution of substrates entering glycogenesis at the level of triose-P such as fructose, over substrates that originated by the Krebs cycle anaplerosis. Another noteworthy feature of spectra from FRU mice was that the enrichment of position 2 was significantly lower than that of position 5 ($P = 0.003$). Assuming complete exchange between water and metabolite hydrogens at the various steps of the glycolytic pathways shown in Figure 1, enrichment of position 2 can never be exceeded by that of position 5. However, if exchange mediated by G6P isomerase is incomplete, enrichment of position 2 will be depleted relative to that of position 5. Our previous studies in fish^{3,8} and in rats⁶ indicate that under conditions of hepatic glycogen synthesis, exchange of body water and position 2 hydrogens through G6P isomerase is substantially incomplete. Because this information was not known for mice, we quantified the extent of (G6P) isomerase exchange based on the enrichment of glycogen positions 2 and 3 following $[\text{U-}^2\text{H}_7]\text{glucose}$ feeding⁶ in a separate study. This study indicates that $64 \pm 1\%$ of G6P position 2 hydrogens underwent exchange during glycogen synthesis. Based on this, a correction factor of 1.57 was applied to the glycogen position 2 enrichments measured in the $^2\text{H}_2\text{O}$ studies.⁶ The

adjusted position 2 enrichments are given alongside the measured ones in Table 1.

Based on the adjusted enrichment of position 2 and the unadjusted enrichments of positions 5 and 6_S, the fractional contributions of direct pathway and/or cycling, indirect pathway from substrates entering at the level of triose-P, and indirect pathway contributions through the Krebs cycle to overnight glycogen appearance were calculated and are provided in Table 1. In FRU mice, most of the overnight glycogen appearance was derived from substrates directly feeding triose-P, which is consistent with glycogenesis from fructose. Direct pathway and/or cycling and Krebs cycle sources accounted for only minor portions. The glycogen synthesis profile of GLU mice was markedly different, with direct pathway + glycogen cycling activities accounting for the bulk of newly appeared glycogen. Moreover, indirect pathway contributions from the Krebs cycle exceeded those from triose-P substrates.

4 | DISCUSSION

Since the concept of hepatic glycogen synthesis through gluconeogenic intermediates, referred to as the indirect pathway, was advanced and validated experimentally,^{9,10} the activity and role of this pathway has been studied under various physiological and pathophysiological conditions.¹¹⁻¹⁴ However, to date, relatively little attention has been paid to the sources of indirect pathway carbons despite the early realization that any gluconeogenic substrate could in principle feed the indirect pathway.¹⁰ Because fructose is initially metabolized to triose-P, the indirect pathway is obligatory for its conversion to glycogen. There is extensive evidence that fructose per se is a potent glycogenic substrate. Substrate balance studies in fasted dogs have shown that intraportally infused fructose results in the rapid synthesis of hepatic glycogen to supraphysiological levels.^{15,16} Intravenous infusion of fructose into healthy humans subjects resulted in an increased flux through uridine diphosphate glucose, indicative of increased glycogen synthase activity.¹⁷ Our observations of triose-P sources being the dominant source of hepatic glycogen synthesis in the FRU mice are fully consistent with these previous studies. For mice given glucose as the sole dietary carbohydrate, direct pathway and/or glycogen cycling was the dominant contributor to overnight glycogen appearance. If hepatic glycogen turnover is incomplete, pre-existing unlabeled glycogen can be converted to [2-²H] glycogen via glycogen cycling. If, on the other hand, the glycogen pool has fully turned over during the interval of ²H₂O administration, then [2-²H]glycogen can only be generated via the direct pathway and glycogen cycling has no effect on its appearance. Under conditions of incomplete glycogen turnover, resolution of direct pathway and glycogen cycling

fluxes requires an independent measurement of net glycogen synthesis, or a complementary glucose tracer such as [U-¹³C] glucose.³

The FRU mice had a sizable contribution of glycogen enriched in position 2 only, indicative of direct pathway metabolism of unlabeled glucose or glycogen cycling activity. Because dietary sources of glucose were absent from the feed of FRU mice, we conclude that glycogen cycling was solely responsible for this activity. In healthy human subjects, glycogen cycling was shown to be increased during infusion of substrate levels of fructose.¹⁹

Our analysis makes several assumptions on substrate availability and metabolism. As indicated in Figure 1, the ²H-enrichment profile of glycogen synthesized from fructose is identical to that formed from glycerol; hence, contributions from these 2 substrates cannot be distinguished. Given that under fed conditions, circulating glycerol levels are low and that glycerol-3-phosphate flux is directed toward fatty acid esterification rather than gluconeogenesis, its contribution to glycogen synthesis is likely to be minimal. Concerning the metabolism of fructose, if its triose-P products initially feed into pyruvate and anaplerotic pathways instead of being directly converted to hexose-P, then these will be indistinguishable from other anaplerotic indirect pathway precursors. The low overall contribution of Krebs cycle sources to glycogen turnover in FRU mice suggests that fructose metabolism by this route was relatively minor. Our unpublished observations of glycogen enrichment from [U-¹³C]fructose also indicate relatively low levels of label randomization using the Krebs cycle. In our initial rodent studies of glycogen enrichment from ²H₂O, exchange of G6P position 2 and body water was assumed to be complete.^{1,2} However, later studies showed substantially incomplete exchange in both fish and rats,⁶ whereas a human study indicated near-complete exchange.²⁰ In this report, we confirmed that G6P position 2 and body water exchange was also incomplete for mice fed normal chow and drinking water supplemented with glucose and fructose. The fraction of position 2 hydrogens exchanged and the resulting correction factor (64%, 1.57) was similar to those previously reported for rats (61%, 1.64).⁶ For the mice experiments, G6P exchange was not measured under precisely the same dietary conditions as the ²H₂O studies. If G6P exchange was altered by the different diets, this would introduce systematic errors to both the corrected position 2 enrichments and flux estimates.

5 | CONCLUSIONS

We demonstrated that the ²H-enrichment distribution of hepatic glycogen from ²H₂O in mice informs the contributions of dietary glucose and fructose to hepatic glycogen synthesis using direct and indirect pathways under natural

feeding conditions. This approach may be useful for furthering our understanding of the relationship between hepatic glycogen metabolism and dietary carbohydrate composition.

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