Sources of Hepatic Glycogen Synthesis in Mice Fed with Glucose or Fructose as the Sole Dietary Carbohydrate

Ivana Jarak[1], Cristina Barosa[2], Fatima O. Martins[2], Joao C. P. Silva[2], Cristiano Santos[2], Getachew Debas Belew[2], Joao Rito[2], Ivan Viegas[1,2], Jose Teixeira[2], Paulo Oliveira[2] and John G. Jones[2,3]*

[1] Centre for Functional Ecology, Department of Life Sciences, University of Coimbra, Portugal

[2]Center for Neurosciences and Cell Biology, University of Coimbra, Portugal

[3]APDP-Portuguese Diabetes Association, Lisbon Portugal

***Address for Correspondence:**

John G. Jones, D.Sc.

Center for Neurosciences and Cell Biology

UC-Biotech, Biocant Park

3060-197, Cantanhede, Portugal

email: john.griffith.jones@gmail.com

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Abstract (243 words)

Purpose: The positional analysis of hepatic glycogen enrichment from deuterated water (^2H_2O) by ^2H NMR has been previously applied 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 were comprised solely of either glucose or fructose.

Methods: Eight glucose- and twelve fructose-fed mice (GLU-mice and FRU-mice) were given ^2H_2O 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 was 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-P 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 GLU-mice was 63±1%. For the indirect pathway, Krebs cycle and triose-P sources contributed 22±1% and 15±1%, respectively. For FRU-mice, glycogen appearance was dominated by triose-P sources (60±2%) with lesser contributions from Krebs cycle (14±2%) and direct and/or glycogen cycling (26±2%).

Conclusions: ^2H NMR analysis of hepatic glycogen ^2H-enrichment from ^2H_2O provides realistic profiles of dietary glucose and fructose contributions to hepatic glycogen synthesis in mice fed with diets containing one or the other sugar as the sole carbohydrate source.

Keywords: gluconeogenesis, deuterated water, indirect pathway, ^2H NMR.

Introduction: In Westernized societies, the surge in obesity and related complications such as Type 2 diabetes and non-alcoholic fatty liver disease has in part been attributed to increased consumption of refined sugar in the form of sucrose or high-fructose corn syrup. Since 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 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 via the indirect pathway (Figure 1). We previously demonstrated in rat models that glycogen synthesis via direct and indirect pathways can be quantified during natural overnight using deuterated water (^2H_2O) (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 via 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 glycogenic metabolism of the fructose component of sucrose. Since dietary glucose sources were more plentiful than that of fructose in these studies[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 was comprised entirely of glucose, then hepatic glycogen synthesis from triose-P sources should be minimal, while direct pathway contributions would dominate. We tested this hypothesis by measuring the sources of hepatic glycogen synthesis in two groups of mice: one group fed a synthetic diet where fructose was the sole carbohydrate component and the other fed a diet with glucose as the sole carbohydrate.

[1] Glucose equivalents from the drinking water sucrose plus additional glucose derived from maltose in the chow.

Methods

Materials: $^{2}H_2O$ at 99.9% enrichment was obtained from Eurisotop, Saint-Aubain, France.

Animal Studies: Animal studies were approved by the University of Coimbra Ethics Committee on Animal Studies (ORBEA) and the Portuguese National Authority for Animal Health (DGAV), 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. They were maintained with a 12h light/12h dark cycle. Upon delivery to the Bioterium, mice were provided a two week interval for acclimation, with free access to water and standard chow, comprising of 60% mixed carbohydrate, 16% protein and 3% lipid. After this period, animals were randomly assigned to two 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 h of the light period for a total time of 16 h. 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 min after gavage using a OneTouch Vita (LifeScan) glucometer and glucose tolerance was assessed by the area under the curve over 0-120 min.

On the ultimate evening of the feeding trial, mice were given an intraperitoneal injection of 99.9% $^{2}H_2O$ with 0.9% w/v NaCl at a dose of 3 grams/100 g body weight at the start of the dark cycle. At the same time, the drinking water was supplemented with 99.9% $^{2}H_2O$ (5% v/v). 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 ºC 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% w/w glucose enriched to 20% with [U-$^2H_7$]glucose, and 15% w/v unlabelled fructose. After *ad libitum* feeding and drinking overnight, the mice were euthanized, and liver glycogen was extracted and analysed by ^2H NMR as described below. The ratio of glycogen enrichment in positions 2 and 3 was used to calculate the correction factor for incomplete glucose-6-phosphate-fructose-6-phosphate exchange as previously described (6).

Glycogen extraction and monoacetone glucose synthesis: Samples were prepared as described previously by Rito at al. (3) Briefly, glycogen was extracted from frozen liver powder by treatment with 30% KOH (2 ml/g of liver) at 70ºC 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, 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 55ºC. The supernatant was lyophilized and mixed with 5 ml ^2H-enriched acetone prepared as described (4) and 4% sulphuric acid enriched to 2% with $^2H_2SO_4$ (v/v). The mixture was stirred overnight at room temperature. The reaction was quenched with water (5 ml, enriched to 2% with ^2H_2O), the pH adjusted with HCl (pH 2.0) and the mixture incubated at 40ºC for 5 hours. The solution pH was adjusted to 8 with $NaHCO_3$ and the samples evaporated to dryness. Monoacetone glucose (MAG) 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).

NMR analysis: Proton-decoupled ^{2}H NMR spectra were obtained with a Varian VNMRS 600 MHz NMR (Agilent) spectrometer equipped with a 3-mm broadband probe. Plasma body water ^{2}H-enrichments were determined from 10 µL of plasma by ^{2}H NMR as described previously (5). MAG samples were dissolved in 90% acetonitrile/10% water and the ^{2}H NMR spectra were acquired at 50 ºC using a 90º pulse and 1.7 s of recycling time (1.6 s of acquisition time and 0.1 s pulse delay). The spectra were processed with 1.0 Hz line-broadening before Fourier transformation. Positional ^{2}H enrichments were determined from the known enrichment of MAG methyl signals that were used as an internal standard. Spectra were analyzed using NUTS PC-based NMR spectral analysis software (Acorn NMR Inc., USA).

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

Equation 1: Direct pathway and/or glycogen cycling = $100 \times (1-H5/H2)$

Equation 2: Indirect pathway sources via Krebs cycle = $100 \times H6_S/H2$

Equation 3: Indirect pathway sources via Triose-P = $100 \times (H5 - H6_S/H2)$

H2 was adjusted for the known incomplete exchange of body water and position 2 hydrogens via glucose-6-phosphate isomerase (6). Since glycogen cycling (glycogen → Glu-1-P ↔ Glu-6-P ↔ Fru-6-P ↔ Glu-6-P ↔ Glu-1-P → UDPG → 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.

Statistics: All results are presented as means ± standard error and comparisons were made by an unpaired t-test (two tailed) performed using Microsoft Excel.

Results

The two 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 GLU-mice and 22.6 ± 0.6 g to 24.6 ± 0.4 g for FRU-mice. The two groups also had equal fasting glucose levels and glucose tolerance at baseline as well as in the fifth and tenth week of feeding (data not shown). Body water enrichments had a tendency to be higher in fructose-fed (FRU-mice) compared to glucose-fed mice (GLU-mice), as shown in Table 1, but the difference was not significant (p = 0.18). Figure 2 shows representative ^{2}H NMR spectra of derivatized glycogen obtained from a FRU-mouse **a**), and a GLU-mouse **b**). The spectra had well-resolved signals with high signal-to-noise ratios from all seven hydrogens attached to the hexose carbon skeleton allowing precise quantification of positional ^{2}H-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 via 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 glycogenic pathways shown in Figure 1, enrichment of position 2 can never be exceeded by that of position 5. We developed an assay for quantifying the fraction of G6P hydrogen 2 exchanged during direct pathway glycogenesis based on the retention of ^{2}H in glycogen position 2 relative to position 3 following [U-$^2H_7$]glucose metabolism (6). Our previous studies in fish (3,8) and in rats (6) indicate that under conditions of hepatic glycogen synthesis, exchange of body water and position 2 hydrogens via glucose-6-phosphate isomerase (G6PI) is substantially incomplete regardless of direct or indirect pathway contributions. This, rather than any analytical artefact, provides a satisfactory explanation for the lower than expected position 2 enrichment. We therefore applied the [U-$^2H_7$]glucose assay to mice and obtained a correction factor (1.57 ± 0.03) that

allowed position 2 enrichment to be adjusted for incomplete G6PI-mediated exchange (6). This value is similar to that reported previously in rats (1.64) (6). The adjusted position 2 enrichments are shown 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 via the Krebs cycle to overnight glycogen appearance were calculated and are are shown in Table 1. In FRU-mice, the majority of overnight glycogen appearance was derived from substrates directly feeding triose-P – 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.

Discussion

Since the concept of hepatic glycogen synthesis via 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). Since 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 UDP-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[2], the ^2H_2O method *per se* cannot resolve direct pathway and glycogen cycling fluxes. This requires an independent measurement of net hepatic glycogen synthesis (7,18) or the integration of ^2H_2O and [U-^{13}C]glucose tracers (3).

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. Since 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 ^{2}H-enrichment profile of glycogen synthesized from fructose is identical to that formed from glycerol, hence contributions from these two substrates cannot be distinguished. Given that under fed conditions, circulating glycerol levels are low and that glycerol-3-phosphate flux is directed towards 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-^{13}C]fructose also indicate relatively low levels of label randomization via the Krebs cycle. In our initial studies of glycogen enrichment from ^2H_2O, 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) while a human study indicated near-

[2] Pre-existing unlabeled glycogen is converted to [2-^{2}H]glycogen via glycogen cycling. When glycogen turnover is complete, new [2-^{2}H]glycogen can only be generated by the direct pathway metabolism of dietary glucose.

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 hence a correction factor was applied to the measured position 2 enrichment. To the extent that this correction factor differs between this setting and those of the synthetic glucose and fructose diets, the corrected position 2 enrichment and fractional flux estimates will have systematic errors.

In conclusion, we demonstrated that the ^{2}H-enrichment distribution of hepatic glycogen from $^{2}H_2O$ in mice informs the contributions of dietary glucose and fructose to hepatic glycogen synthesis via 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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Footnotes:

1. Glucose equivalents from the drinking water sucrose plus additional glucose derived from maltose in the chow.
2. Pre-existing unlabeled glycogen is converted to [2-^{2}H]glycogen via glycogen cycling. When glycogen turnover is complete, new [2-^{2}H]glycogen can only be generated by the direct pathway metabolism of dietary glucose.

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Figure Legends

Figure 1: Schematic of glycogen synthesis from fructose and glucose and positional enrichment of glycogen from $^{2}H_2O$. For clarity, some metabolic intermediates have been omitted. Glycogen derived via direct pathway metabolism of glucose (Glucose → Glu-6-P → Glu-1-P → UDPG → Glycogen) or undergoing cycling following hydrolysis to Glu-1-P (blue arrow) will be enriched in position 2 (**A**). Glycogen derived from triose-P sources, including fructose, will be enriched in positions 2 and 5 (**B**). Glycogen derived from pyruvate (which may originate from glucose metabolized peripherally via 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 (**C**).

Figure 2: ^{2}H NMR spectra of monoacetone glucose derived from liver glycogen of a mouse fed with a diet where fructose was the sole carbohydrate **a)** and a diet where glucose was the sole carbohydrate **b)**. The numbers above the signals indicate their glucosyl positional origin.

Table 1: Glycogen and body water ^{2}H-enrichments and fractional contributions of various sources to overnight glycogen appearance following $^{2}H_2O$ administration for mice fed with diets whose carbohydrate component consisted either entirely of fructose or of glucose. Data are shown as means with standard errors in parentheses below each value. *Position 2 enrichment after correction for incomplete exchange of ^{2}H between water and glucose-6-P position 2.

Diet		**Glycogen positional enrichments and body water (BW) enrichment**								**Glycogen appearance sources (%)**		
	1	2	2*	3	4	5	6R	6S	BW	Direct and/or cycling	Indirect-Triose-P	Indirect-Krebs cycle
Fructose (n=12)	0.67 (0.09)	1.43 (0.04)	2.26 (0.07)	1.32 (0.04)	1.52 (0.04)	1.66 (0.05)	0.35 (0.02)	0.32 (0.01)	2.70 (0.01)	26 [a] (2)	60[b] (2)	14[c] (1)
Glucose (n=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)

[a] $p = 3 \times 10^{-10}$ compared to glucose; [b] $p = 1 \times 10^{-12}$ compared to glucose; [c] $p = 2 \times 10^{-4}$ compared to glucose.

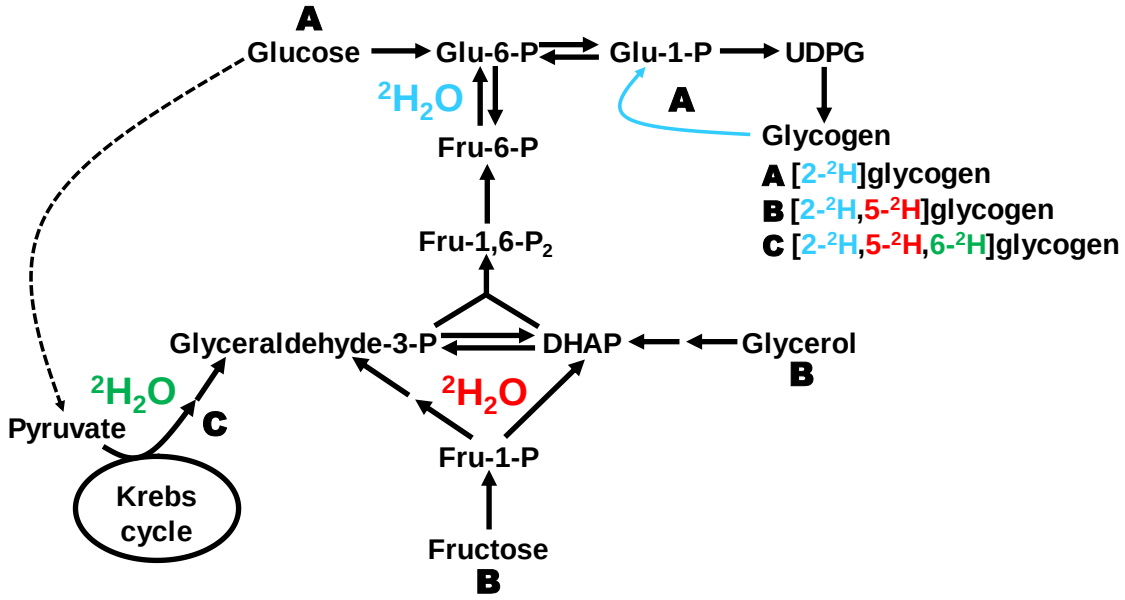

Figure 1

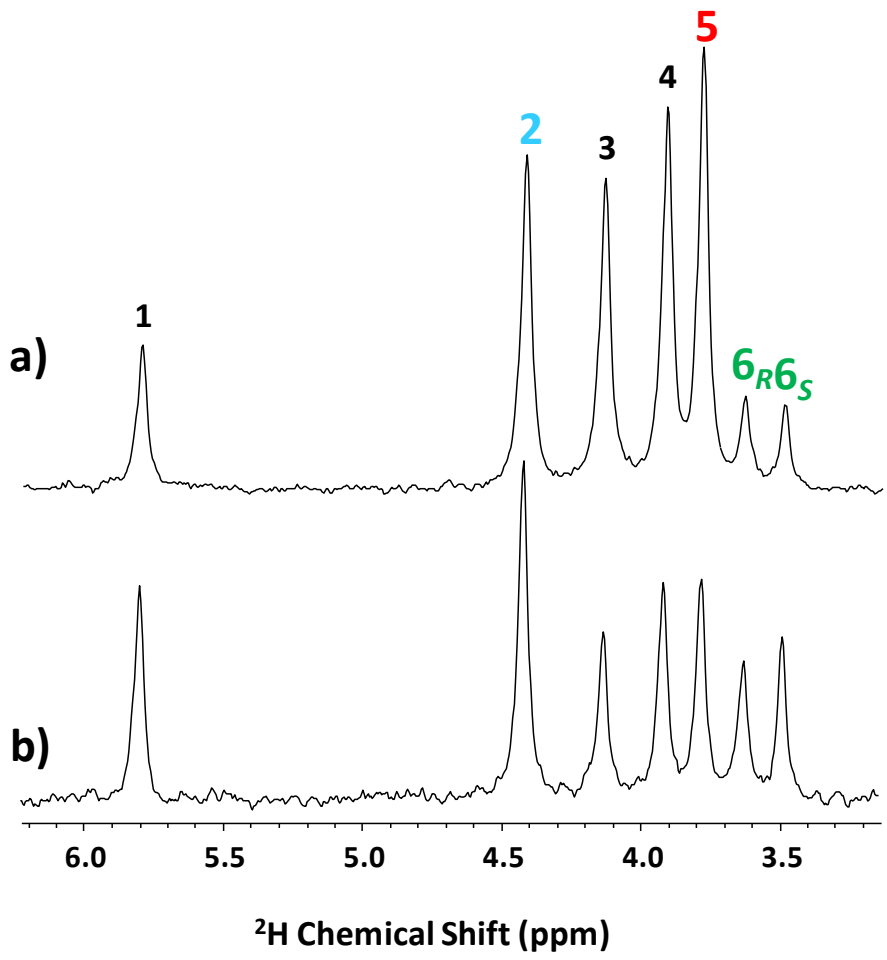

Figure 2