Original Article

Noninvasive Analysis of Hepatic Glycogen Kinetics Before and After Breakfast with Deuterated Water and Acetaminophen

John G. Jones,[1] Ana Fagulha,[2] Cristina Barosa,[1] Margarida Bastos,[2] Luisa Barros,[2] Carla Baptista,[2] M. Madalena Caldeira,[3] and Manuela Carvalheiro[2]

The contributions of hepatic glycogenolysis to fasting glucose production and direct pathway to hepatic glycogen synthesis were quantified in eight type 1 diabetic patients and nine healthy control subjects by ingestion of ^2H_2O and acetaminophen before breakfast followed by analysis of urinary water and acetaminophen glucuronide. After overnight fasting, enrichment of glucuronide position 5 relative to body water (G5/body water) was significantly higher in type 1 diabetic patients compared with control subjects, indicating a reduced contribution of glycogenolysis to glucose production (38 ± 3 vs. 46 ± 2%). Following breakfast, G5/body water was significantly higher in type 1 diabetic patients, indicating a smaller direct pathway contribution to glycogen synthesis (47 ± 2 vs. 59 ± 2%). Glucuronide hydrogen 2 enrichment (G2) was equivalent to body water during fasting (G2/body water 0.94 ± 0.03 and 1.02 ± 0.06 for control and type 1 diabetic subjects, respectively) but was significantly lower after breakfast (G2/body water 0.78 ± 0.03 and 0.82 ± 0.05 for control and type 1 diabetic subjects, respectively). The reduced postprandial G2 levels reflect incomplete glucose-6-phosphate–fructose-6-phosphate exchange or glycogen synthesis from dietary galactose. Unlike current measurements of human hepatic glycogen metabolism, the ^2H_2O/acetaminophen assay does not require specialized on-site clinical equipment or personnel. *Diabetes* 55:2294–2300, 2006

Hepatic glycogen is the principal short-term reserve for circulating glucose in humans and therefore plays an important role in glucose homeostasis. Patients with poorly controlled type 1 diabetes accumulate poor hepatic glycogen stores during feeding, and these sustain limited rates of endogenous glucose production during fasting. Consequently, the maintenance of fasting euglycemia in response to a drop in blood glucose levels becomes more dependent on the activation of gluconeogenesis by counterregulatory mechanisms. For many type 1 diabetic subjects this functions poorly, thereby increasing the patient's vulnerability to a hypoglycemic episode. Thus, insufficient rates of hepatic glycogen storage and hydrolysis may contribute to the development of fasting hypoglycemia. Hepatic glycogen is synthesized by two distinct processes: the classical direct pathway from intact glucose units and an indirect pathway involving 3-carbon intermediates such as pyruvate (1–3). With current human tracer methodologies, true hepatic indirect pathway activity is indistinguishable from Cori cycle metabolism of glucose since 3-carbon intermediates are common to both processes. Also, nonglucose precursors such as glycerol or gluconeogenic amino acids can feed the 3-carbon pool and contribute to indirect pathway flux. In healthy subjects, the direct pathway accounts for the bulk of hepatic glycogen synthesis (4,5), but in type 1 diabetic patients, its contribution is reduced to ~50% (6–8).

In type 1 diabetic patients, some key deficiencies of hepatic glycogen fluxes can be restored by intensive dietary and insulin therapy. After establishing good long-term metabolic control as a result of tailored multiple insulin injections, type 1 diabetic patients were able to convert dietary carbohydrate to glycogen at comparable rates to healthy subjects (8). Also, hepatic glycogenolysis rates and the fractional contribution of glycogenolysis to endogenous glucose production after overnight fasting were restored to normal values (8). However, this intervention did not increase the fraction of glycogen synthesized by the direct pathway to that of healthy control subjects (8). These and other studies (6,9) demonstrate that assessment of hepatic glycogen kinetics could be useful for evaluating and guiding therapies of glycemic control in type 1 diabetic patients. Currently, rates of hepatic glycogen synthesis and hydrolysis are obtained by observing the natural abundance ^{13}C glycogen signal with a whole-body magnetic resonance system (6,8,10–12). While this method provides a direct assessment of hepatic glycogen levels, it is only available in a few research centers around the world. Measurement of direct and indirect pathway activities involve the ingestion of 10 g [1-^{13}C]glucose in a breakfast meal and analysis of ^{13}C-enrichment in plasma glucose and urinary glucuronide (13). The tracer is relatively expensive and both blood and urine need to be collected.

From the [1]NMR Research Unit, Department of Biochemistry and Center for Neurosciences and Cell Biology, University of Coimbra, Coimbra, Portugal; the [2]Department of Endocrinology, University Hospital of Coimbra, Coimbra, Portugal; and the [3]Department of Chemistry, University of Coimbra, Coimbra, Portugal.

Address correspondence and reprint requests to John G. Jones, DSc, NMR Research Unit, Department of Biochemistry and Center for Neurosciences and Cell Biology, Faculty of Sciences and Technology, University of Coimbra, 3001-401 Coimbra, Portugal. E-mail: jones@cnc.cj.uc.pt.

DMF, dimethylformamide; F6P, fructose-6-phosphate; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; MAG, monoacetone glucose; NMR, nuclear magnetic resonance; UDPG, uridine diphosphate glucose.

Received for publication 6 March 2006 and accepted in revised form 19 May 2006.

DOI: 10.2337/db06-0304

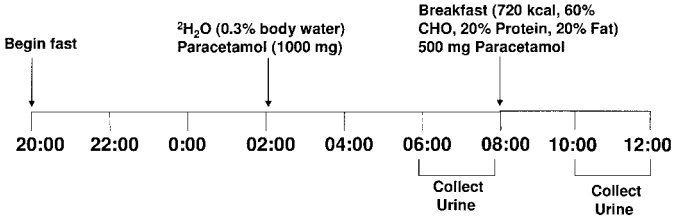

FIG. 1. ^2H_2O and acetaminophen administration and urine collection protocol.

We present a practical and noninvasive method that provides information on the direct/indirect pathway of hepatic glycogen synthesis during feeding and the contribution of glycogenolysis to endogenous glucose production during fasting. The assay involves the oral administration of deuterated water (^2H_2O) and acetaminophen followed by the analysis of deuterium (^2H) enrichment of urinary acetaminophen glucuronide before and after a breakfast meal (Fig. 1). Glucuronide is derived from hepatic uridine diphosphate glucose (UDPG), the immediate precursor of glycogen synthesis (Fig. 2). As a result of exchange between glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) catalyzed by G6P-isomerase (14), UDPG synthesized via the direct pathway will be enriched with ^2H in position 2. If G6P-F6P exchange is complete, hydrogen 2 of UDPG will be enriched to the same level as body water. UDPG synthesized via 3-carbon precursors (i.e., indirect pathway or gluconeogenesis) will be enriched in both 2 and 5 positions due to exchange/addition of water and metabolite hydrogens at triose and hexose phosphate levels. Therefore, the contribution of the direct relative to the indirect pathways of hepatic glycogen synthesis can be estimated by the ratio of glucuronide ^2H-enrichment in position 5 relative to position 2 (G5/G2).

During fasting, when glycogen is hydrolyzed to glucose, there remains a residual flux through UDPG and glucuronidation pathways. Due to the rapid interchange of hepatic G6P, glucose-1-phosphate (G1P), and F6P pools, the ^2H-enrichment pattern of G6P diverted for glucuronide synthesis is equivalent to that of plasma glucose. Glycogenolysis will generate hexose-P with ^2H-enrichment in position 2 only, while gluconeogenesis will generate hexose-P enriched in positions 2 and 5. Therefore, the fraction of glucose output derived from glycogenolysis can be estimated from G5/G2 of the fasting glucuronide (15). Glucuronide G5/G2 values can be conveniently determined by ^2H nuclear magnetic resonance (NMR) following derivatization to monoacetone glucose (MAG) (15). Where applicable, the use of menthol in the form of peppermint oil as the glucuronidation probe can further simplify the procedure since urinary menthol glucuronide can be analyzed directly after a simple purification procedure (16).

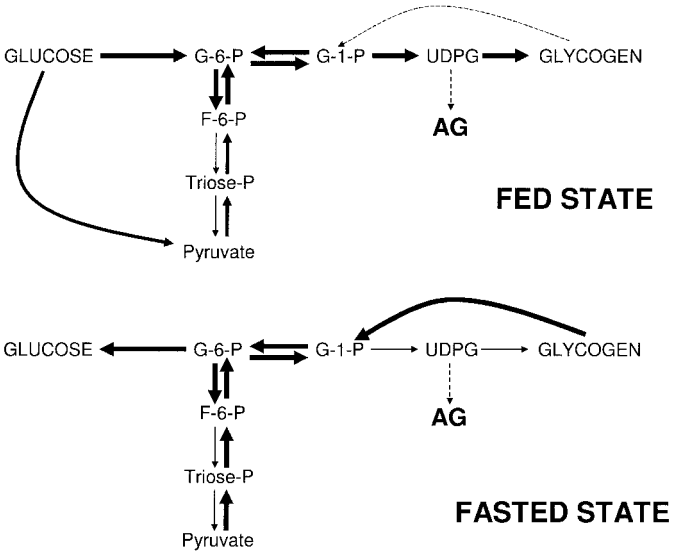

FIG. 2. Hepatic glucose-glycogen fluxes and sampling by acetaminophen glucuronide (AG) during fed and fasted states.

RESEARCH DESIGN AND METHODS

Nine healthy subjects (3 women and 6 men, age 21 ± 1 years, weight 67 ± 13 kg, HbA_{1c} [A1C] 4.9 ± 0.2%) and eight type 1 diabetic subjects (3 women and 5 men, age 20 ± 1 years, weight 65 ± 8 kg, A1C 8.1 ± 1.6%, mean duration of diabetes 10 ± 2 years) were studied. Exclusion criteria included use of tobacco, antihypertensive medications, systemic steroids, and renal or hepatic dysfunction. Subjects were admitted into the hospital and began fasting at 2000 following a standard dinner. At 0200, each subject ingested 1,000 mg acetaminophen and 3.0 g/kg body water of ^2H_2O as a 40% solution in noncarbonated spring water (~350 ml total volume) imbibed over 15–20 min. For the remainder of the study, the subject drank water containing 0.3% ^2H_2O to maintain body water enrichment. After collection of urine from 0600 to 0800, the subject ingested a standardized breakfast conforming to American Diabetes Association guidelines (720 kcal, 60% carbohydrate, 20% protein, and 20% fat) and 500 mg acetaminophen. Subjects voided at 1000, and urine was collected from 1000 to 1200.

Sample processing and NMR spectroscopy. Acetaminophen glucuronide was converted to MAG as described (17). Proton-decoupled MAG ^2H NMR spectra were acquired at 11.75 T with a Varian Unity 500 system equipped with a 5-mm broadband probe (Varian, Palo Alto, CA). Spectra were obtained at 50°C without field-frequency lock with a 90° pulse angle, a sweep width of 10 ppm, an acquisition time of 1.6 s, and no pulse delay. Between 8,000 and 30,000 free induction decays were acquired per sample for collection times of 3.5–13.3 h. The summed free induction decays were processed with 0.5–1.0 Hz line-broadening before Fourier transform. Fully relaxed ^1H NMR spectra were obtained under the same conditions with presaturation of the acetonitrile signal. A pulse width of 45°, acquisition time of 3 s, and a delay of 16 s were used. For each ^1H spectrum, 4–16 free induction decays were acquired. ^2H enrichment of urine water was analyzed by ^2H NMR as previously described (18). All NMR spectra were analyzed using the curve-fitting routine supplied with the NUTS PC-based NMR spectral analysis program (Acorn NMR, Fremont, CA).

Quantification of glucuronide hydrogen 2 absolute ^2H-enrichment. Absolute ^2H enrichment of glucuronide hydrogen 2, hereafter defined as G2, was obtained by calculating the moles of ^1H and ^2H nuclei in position 2 of MAG. The amount of [2-^1H]MAG was obtained from the ratio of dimethylformamide (DMF) methyl (^1H DMF) and MAG hydrogen 2 (^1H MAG) proton signals multiplied by the amount of ^1H DMF present (9.58 μmol) and corrected for the 1:3 stoichiometry of the MAG and DMF methyl protons (equation 1 below). Likewise, the amount of [2-^2H]MAG was obtained from the ratio of DMF methyl (^2H DMF) and the MAG hydrogen 2 (^2H MAG) deuterium signals multiplied by the amount of ^2H DMF present (0.46 μmol) and the 1:3 stoichiometry factor (equation 2 below). In addition, DMF ^2H signals are partially saturated under the NMR acquisition conditions due to long relaxation times (T_1 = 2–4 s), while those of MAG are fully relaxed (T_1 = 175–250 ms). To account for partial saturation, each ^2H DMF methyl signal intensity was multiplied by a correction factor, obtained by quantifying the change in the DMF ^2H signal intensity relative to that of a 3% [2-^2H]MAG standard obtained under ambient and fully relaxed conditions: *1*) [2-^1H]MAG (μmol) = 9.58 × 0.333 × (^1H DMF/^1H MAG); *2*) [2-^2H]MAG (μmol) = 0.46 × 0.333 × (^2H DMF/^2H MAG); *3*) %^2H enrichment of MAG hydrogen 2 (G2) = [2-^2H]MAG/([2-^2H]MAG + [2-^1H]MAG) × 100.

G2 was calculated from the quantities of ^1H and ^2H as shown in equation 3. ^2H fractional enrichment determinations were performed with both DMF methyl signals and the mean of both values is reported.

Quantification of hepatic glycogen fluxes. From urine collected before breakfast, the contribution of gluconeogenesis and glycogenolysis to hepatic glucose production was estimated from the enrichment of glucuronide position 5 (G5) relative to that of body water (G5/body water) as shown by equations 1 and 2: *4*) glucose production from glycogenolysis (%) = 1-(G5/body water) × 100 and *5*) glucose production from gluconeogenesis (%) =

(G5/body water) × 100. G5 was derived by multiplying G2 by the ratio of signal 5 to signal 2 of the MAG ^{2}H NMR spectrum.

From urine collected after breakfast, the contribution of direct and indirect pathways to hepatic glycogen synthesis was estimated from G5/body water, as shown by equations 1 and 2: *6*) glycogen synthesis via direct pathway (%) = 1-(G5/body water) × 100 and *7*) glycogen synthesis via indirect pathway (%) = (G5/body water) × 100.

Unless indicated otherwise, all data are presented along with means and SDs. Differences between means of experimental data obtained from healthy and type 1 diabetic subjects were evaluated by a two-tailed, heteroscedastic Student's *t* test. *P* values of <0.05 were considered significant.

RESULTS

For both subject groups, a constant level of body water enrichment was achieved before and after the breakfast meal (Table 1). Also, enrichment of glucuronide hydrogen 2 (G2) during fasting approached that of body water indicating quantitative incorporation of body water ^{2}H into position 2 of G6P. For the postprandial glucuronide of both study groups, G2 was significantly lower than that of the fasting glucuronide and was only ~80% of body water enrichment.

^{2}H NMR spectra of MAG derived from acetaminophen glucuronide generated well-resolved ^{2}H MAG resonances with high signal-to-noise ratios (Fig. 3). Each signal represents ^{2}H-enrichment in a specific position of glucuronide and the signal intensities are proportional to the enrichment of ^{2}H at that site. ^{2}H-NMR spectra derived from the prebreakfast glucuronide of healthy control subjects were consistent with those reported in a study of overnight-fasted healthy control subjects (15). The position 2 signal had the highest intensity, reflecting the near-equivalent ^{2}H-enrichment of UDPG hydrogen 2 and body water. Other MAG ^{2}H-signals corresponding to positions 3, 4, and 5 of glucuronide were less intense, indicating dilution of ^{2}H-enrichment at these sites by G6P derived from unlabeled hepatic glycogen. The intensity of signal 5 relative to signal 2 corresponds to the ^{2}H-enrichment ratio of positions 5 and 2 (G5/G2) and provides an estimate of the relative contribution of gluconeogenesis and glycogenolysis to G6P synthesis (10,19). While the mean G5/G2 was significantly higher for the type 1 diabetic group compared with healthy control subjects, the ranges (0.52–0.70 for type 1 diabetic and 0.49–0.61 for healthy control subjects) overlapped considerably. For both healthy and type 1 diabetic subjects, estimates of glycogenolysis derived from G5/body water enrichment were equal to those obtained from that of glucuronide G5/G2 as expected from the equivalence of G2 and body water enrichment. As was previously observed with plasma glucose enrichment distributions from ^2H_2O in healthy subjects and cirrhotic patients, the hydrogen 4 and 5 signals of MAG had equal intensities indicating that G6P hydrogens 4 and 5 were enriched to equivalent levels from ^2H_2O (20). Also, hydrogen 3 enrichment was significantly lower than hydrogen 5 (0.09 ± 0.01 vs. 0.14 ± 0.01, $P < 0.0002$ for control subjects; and 0.10 ± 0.01 vs. 0.16 ± 0.01, $P < 0.0003$ for type 1 diabetic patients), indicating that exchange of 1*R*-dihydroxyacetone phosphate (the precursor of G6P hydrogen 3) with body water was incomplete. ^{2}H NMR signals were not observed for the six pro-R and six pro-S hydrogens of MAG since these are obtained from the reduction step in the derivatization procedure and are therefore not enriched above background level (15). Estimates of fractional gluconeogenesis and glycogenolysis derived from fasting G5/G2 of healthy subjects indicate that half (46 ± 2%) of fasting glucose production was derived from glycogenoly-

TABLE 1
Summary of ^{2}H-enrichment data from urinary glucuronides (Glc) and body water (bw) for healthy control and type 1 diabetic patients

		Prebreakfast glucuronide				Postbreakfast glucuronide			
	A1C (%)	^{2}H bw (%)	Glc H2/bw	Glc H5/H2	Glc H5/bw	^{2}H bw (%)	Glc H2/bw	Glc H5/H2	Glc H5/bw
Control subjects									
1	5.0	0.30	0.96	0.61	0.59	0.29	0.75	0.51	0.38
2	4.7	0.29	0.92	0.59	0.55	0.31	0.77	0.52	0.41
3	4.9	0.26	0.92	0.50	0.46	0.26	0.76	0.57	0.43
4	5.0	0.29	0.90	0.56	0.50	0.30	0.66	0.49	0.33
5	5.1	0.24	0.81	0.57	0.46	0.26	0.68	0.54	0.37
6	N.D.	0.30	0.90	0.49	0.45	0.28	0.83	0.51	0.43
7	N.D.	0.26	0.94	0.50	0.47	0.25	0.92	0.53	0.49
8	N.D.	0.25	1.09	0.52	0.57	0.26	0.76	0.58	0.44
9	4.7	0.23	1.05	0.49	0.51	0.23	0.86	0.50	0.43
Mean ± SE	4.9 ± 0.2	0.27 ± 0.01	0.94 ± 0.03	0.54 ± 0.02	0.54 ± 0.02	0.27 ± 0.03	0.78 ± 0.03*	0.53 ± 0.03	0.41 ± 0.02
Type 1 diabetic subjects									
1	6.1	0.26	0.88	0.65	0.57	0.28	0.77	0.69	0.53
2	5.6	0.25	0.98	0.69	0.68	0.25	0.62	0.74	0.46
3	8.5	0.29	0.68	0.63	0.43	0.27	0.82	0.58	0.48
4	8.1	0.28	1.06	0.66	0.70	0.26	0.95	0.59	0.56
5	8.0	0.27	1.05	0.55	0.58	0.28	0.74	0.54	0.40
6	9.5	0.24	1.28	0.53	0.68	0.27	0.84	0.61	0.51
7	10.6	0.26	1.04	0.58	0.61	0.27	0.82	0.60	0.49
8	8.5	0.27	1.14	0.61	0.70	0.27	1.05	0.75	0.78
Mean ± SE	8.1 ± 1.6	0.27 ± 0.01	1.02 ± 0.06	0.61 ± 0.02†	0.62 ± 0.03†	0.27 ± 0.02	0.82 ± 0.05‡	0.64 ± 0.03†	0.53 ± 0.02†

* Significantly lower than prebreakfast value, $P < 0.001$. †Significantly higher than control value, $P < 0.05$. ‡Significantly lower than prebreakfast value, $P < 0.05$.

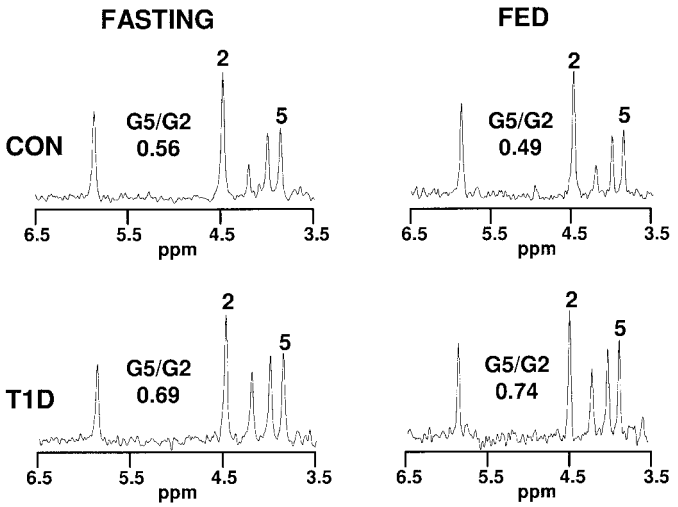

FIG. 3. ^{2}H NMR spectra of the monoacetone glucose derivative of acetaminophen glucuronide with the position 5 and position 2 NMR signals indicated by the number above each signal. Also shown are the G5/G2 enrichment ratios calculated from the relative intensities of these signals. Spectra were obtained from a healthy control subject (CON) before and after breakfast (fasting and fed, respectively). Below is the corresponding fasting and fed NMR spectra from a patient with type 1 diabetes (T1D).

sis. These observations are in good agreement with both gas chromatograph–mass spectrometer and NMR measurements of healthy individuals given similar dosages of ^2H_2O and fasted for similar intervals (20–22). In type 1 diabetic patients, glycogenolysis contributed a significantly smaller fraction of fasting glucose production (38 ± 2%). This observation is consistent with in vivo ^{13}C MR measurements that showed a reduced contribution of glycogenolysis to endogenous glucose production in resting type 1 diabetic subjects compared with healthy control subjects (9).

For both subject groups, glucuronide obtained after breakfast had a ^{2}H-relative enrichment distribution that was remarkably similar to that obtained before breakfast, as shown by the examples in Fig. 3. For any given subject, fed and fasting G5/G2 were closely matched and the relative ^{2}H-signal intensities of hydrogens 3, 4, and 5 of fed and fasted glucuronide were almost identical. For the type 1 diabetic group, enrichment of glucuronide hydrogen 1 relative to hydrogen 2 (G1/G2) tended to be higher in the fed compared with fasted state (0.84 ± 0.11 vs. 0.74 ± 0.09, $P = 0.07$), whereas for healthy control subjects, G1/G2 ratios of fed and fasted glucuronide were not different (0.73 ± 0.04 and 0.74 ± 0.06, respectively).

Postabsorptive glucuronide G5/body water enrichment ratios were significantly different between healthy and type 1 diabetic subjects (see Table 1), indicating differences in the fractional contributions of direct and indirect pathways to hepatic glycogen synthesis. For healthy subjects, the majority of hepatic glycogen (59 ± 2%) was synthesized via the direct pathway, while in type 1 diabetic patients, the direct pathway accounted for a significantly smaller fraction of glycogen synthesis (47 ± 2%, $P < 0.05$ versus control subjects). The G5/body water enrichment ratio was significantly less than G5/G2 under fed conditions, due to G2 being only ~80% that of body water. From this information, the direct pathway contribution can be resolved into two components: one where the hexose moiety underwent G6P-F6P exchange en route to glycogen synthesis and the other where this exchange did not occur. The fraction of glycogen synthesized via these routes and from the indirect pathway is shown in Fig. 4. Our results suggest that for both subject groups, a sizable fraction of direct pathway flux (37 ± 3% for control and 38 ± 5% for type 1 diabetic patients) did not participate in G6P-F6P exchange.

DISCUSSION

Quantifying sources of fasting glucose production by ^2H_2O-glucuronide analysis. Steady-state enrichment of plasma glucose or glucuronide from ingested ^2H_2O is the result of three processes. First, the equilibration of ingested ^2H_2O with bulk body water; second, the various exchange or addition reactions that transfer ^{2}H from body water to precursor metabolites; and third, the turnover of the product metabolite pool. ^2H_2O is equilibrated with body water at 3–4 h (22) and the glucuronide was sampled from 4 to 6 h after ingestion, so the body water ^{2}H is assumed have reached constant enrichment during the time that the initial glucuronide sample was harvested. While the exchange reactions that transfer ^{2}H from water to metabolic intermediates are relatively rapid, there is the possibility of discrimination against ^{2}H incorporation into certain metabolite sites due to kinetic isotope effects (23). Although incorporation of ^{2}H into position 2 of glyceraldehyde-3-P (the precursor for G6P hydrogen 5) does not experience isotopic discrimination (24), the rate of incorporation of ^{2}H into position 2 of G6P via phosphoglucoisomerase is slower than that of ^{1}H due to a primary kinetic isotope effect (23). However, extensive equilibration of G6P and F6P minimizes any isotopic discrimination against the incorporation of ^{2}H into position 2 (23). Compared with plasma glucose, hepatic UDPG and glucuronide are small metabolite pools that are rapidly turning over. As a result, glucuronide reaches isotopic steady-state (defined as the time for the glucose or glucuronide hydrogen 2 enrichment to reach a constant level) at an earlier time than plasma glucose. Insulin resistance or diabetes may result in an increased plasma glucose pool size, possibly prolonging the time for plasma glucose to reach steady-state enrichment. Plasma glucose can be sampled instantaneously, but with glucuronide there is a 30- to 60-min lag time between its formation in the liver and clearance into urine (25). Also, glucuronide is sampled over a period of several hours during which G5/G2 ratios may alter significantly due to changes in physiology, for example the depletion of hepatic glycogen during fasting. In healthy subjects who were given ^2H_2O and fasted overnight, the plasma glucose hydrogen 5–to–hydrogen 2 enrichment ratio (H5/H2) increased by ~10% between the 12th and 14th hour of fasting (22). In another ^2H_2O study, plasma glucose H5/H2 increased by 14% in healthy subjects and 13% for type 2 diabetes patients from the 14th to the 16th hour of fasting (10). Direct and indirect pathway contributions to glycogen synthesis also change over the initial few hours after feeding (4,7). In light of these observations, a 2-h urine collection time represents a compromise between obtaining sufficient urinary glucuronide for NMR analysis and providing reasonable temporal resolution for defining fractional gluconeogenesis or glycogen synthesis in relation to the time of fasting or feeding.

Glycogen metabolism and glucuronide synthesis are both located in the pericentral region of the hepatic lobule, whereas gluconeogenic activity is highest in the periportal

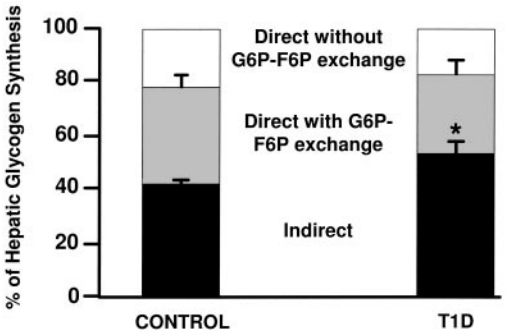

FIG. 4. Sources of hepatic glycogen synthesis in a group of healthy control subjects and a group of type 1 diabetic patients (T1D) as estimated from the fed glucuronide G5/G2 and G5/body water ratios. The black portion represents the indirect pathway contribution, the gray portion represents the contribution from direct pathway with G6P-F6P exchange, and the white portion represents the contribution from direct pathway with no G6P-F6P exchange. Data represent mean values for each group, and the error bar represents the SE.

region (26,27). On this basis, G1P derived from glycogenolysis might be expected to be more available for glucuronide synthesis compared with glucose-1-phosphate derived from gluconeogenesis, resulting in a higher fractional contribution of glycogenolysis to glucuronide synthesis compared with that for hepatic glucose production. In the ^2H_2O experiment, this would result in a glucuronide G5/G2 ratio that was less than that of plasma glucose. Other mechanisms that could result in different glucuronide and plasma glucose enrichments include glucose production from extrahepatic tissues. To date, direct comparisons of plasma glucose and glucuronide ^2H-enrichments from ^2H_2O have been few but equivalent ^2H-enrichment ratios for the two metabolites were reported for healthy control subjects (15). Also, the glucuronide G5/G2 ratios of healthy subjects from this study are consistent with previous gas chromatograph–mass spectrometer and NMR measurements of plasma glucose H5/H2 from healthy subjects given ^2H_2O under similar conditions (14,15,20,22).

Comparison of ^2H_2O and glucose tracers for measuring direct and indirect pathway contributions to glycogen synthesis. Estimates of direct pathway contributions using ^{13}C, ^{14}C, or ^3H tracers have been made during hyperglycemic clamp conditions where the glucose tracer is infused into a peripheral vein and also during mixed meals where the glucose tracer is ingested with the meal. In each case, the specific activity or enrichment of plasma glucose and urinary glucuronide are measured and the extent of label dilution in glucuronide versus plasma glucose (with corrections for tracer recycling) provides a measure of direct and indirect pathways. When [1-^{13}C]glucose was ingested during a mixed meal, direct pathway contributions of 49 ± 7 and 30 ± 8% were reported for control and type 1 diabetic subjects, respectively (8). In a study of healthy subjects that were given [1-^{13}C]glucose by infusion after a breakfast meal, direct pathway estimates of 60–70% were reported (4). From the study of Stingl et al. (28), where dilution of infused [5-^{3}H]glucose in glucuronide versus plasma glucose was measured, the direct pathway accounted for 54–63% of glycogen synthesis in healthy subjects under hyperglycemic clamp conditions. Our direct pathway estimate of 59% for healthy subjects is more closely matched to those measurements where the glucose tracer was infused rather than where it was ingested with the meal.

In the metabolic model representing the fed state (Fig. 2) it is assumed that glucose is the sole carbohydrate substrate, exchange between G6P and F6P is complete, and there is no significant glycogen cycling or transaldolase activity. When these assumptions are unmet, estimates of direct and indirect pathway contributions derived from ^2H_2O are modified differently to those derived from glucose tracers, as summarized in Table 2. [1-^{13}C] or [5-^{3}H]glucose measurements are unaffected by G6P-F6P exchange because this does not redistribute or dilute either tracer. In contrast, the glucuronide ^2H-enrichment distribution from ^2H_2O is sensitive to the extent of G6P-F6P exchange. If exchange is incomplete, G2 is decreased relative to body water and to G5 resulting in an increased G5/G2 ratio and an underestimate of the direct pathway contribution. Under these conditions, G5/body water enrichment provides correct estimates for direct/indirect pathway contributions. Utilization of an alternative carbohydrate source such as galactose will also modify flux estimates from both ^2H_2O and labeled glucose tracers. Galactose is metabolized to UDPG via galactose-1-P and UDP-galactose. No ^2H is incorporated from ^2H_2O into the hexose skeleton during these steps. Therefore, this activity will systematically dilute ^2H-enrichment of all positions resulting in reduced G2/body water and G5/body water ratios but no modification of G5/G2. Therefore, to the extent that galactose contributes to UDPG synthesis, measurement of G5/body water enrichment will overestimate the contribution of the direct pathway. With [1-^{13}C] or [5-^{3}H]glucose, galactose metabolism will dilute the enrichment or specific activity of UDPG, resulting in an underestimate of direct pathway contribution. Glycogen cycling will also modify glucuronide ^2H-enrichment distribution in positions 5 and 2 (28). Inflow of unlabeled hexose units from glycogen hydrolysis results in a systematic dilution of ^2H-enrichment in all sites except for position 2. Glycogen→G1P↔G6P↔F6P↔ G6P↔G1P→UDPG will generate UDPG enriched in hydrogen 2 assuming complete exchange between G1P, G6P, and F6P. Therefore, both G5/G2 and G5/body water enrichment ratios are reduced, resulting in an overestimate of the direct pathway contribution to hepatic glycogen synthesis. With [1-^{13}C]glucose, glycogen cycling dilutes the label at the level of UDPG resulting in an underestimate of the direct pathway contribution to glycogen synthesis. This is also true for the [5-^{3}H]glucose tracer (28). Finally, transaldolase activity exchanges the bottom triose unit of F6P with glyceraldehyde-3-phosphate. This provides an additional mechanism for enriching hydrogen 5 from ^2H_2O or for depleting the specific activity of [5-^{3}H]hexose, therefore with both tracers, the direct pathway contribution is underestimated (28). With [1-^{13}C]glucose, this process does not influence glucuronide C1 enrichment but may slightly increase the enrichment of C6, resulting in a minor underestimation of the direct pathway fraction.

We observed that enrichment of postabsorptive glucuronide was significantly less than that of body water (i.e., G2/body water ~0.8). That this could be an artifact of the NMR measurement is unlikely since G2/body water ratios of ~1.0 were quantified from the fasting glucuronide. As discussed above, UDPG synthesis from galactose and incomplete G6P-F6P exchange could both contribute to G2 being less that that of body water. The breakfast meal contained a significant source of galactose in the form of 400 ml reduced-fat milk. Following release from lactose and uptake into splanchnic blood, essentially all galactose is metabolized in liver compared with only approximately

TABLE 2
Effects of incomplete G6P-F6P exchange, glycogen cycling and galactose metabolism on estimates of the direct pathway contributions to hepatic glycogen synthesis obtained with glucose and ^2H_2O tracers

	[1-^{13}C] or [5-^{3}H]glucose	G5/G2 ratio from ^2H_2O	G5/H_2O ratio from ^2H_2O
Incomplete G6P-F6P exchange	No effect	Direct pathway fraction underestimated	No effect
Glycogen cycling	Direct pathway fraction underestimated	Direct pathway fraction overestimated	Direct pathway fraction overestimated
Galactose metabolism	Direct pathway fraction underestimated	No effect	Direct pathway fraction overestimated
Transaldolase exchange	Direct pathway fraction underestimated*	Direct pathway fraction underestimated	Direct pathway fraction underestimated

*Only to a very minor degree with [1-^{13}C]glucose.

one-fifth of the total glucose carbohydrate equivalents. The amount of milk provided could supply sufficient galactose to systematically dilute the hepatic UDPG enrichment by at least 20% and account for the observed G2/body water enrichment ratio. Four hundred milliliters of reduced fat milk contains ~20.8 g of lactose or 10.4 g galactose for UDPG synthesis. The total breakfast carbohydrate load was 113 grams, of which ~103 grams were glucose units. Assuming that ~20 grams of glucose was taken up by the liver and converted to UDPG, then the contribution of UDPG flux from galactose could be as high as 10.4/30.4 = 34%. However, similar G2/body water ratios (~0.83) were also found during a hyperglycemic clamp, where glucose is the only carbohydrate source for hepatic glycogen synthesis and UDPG synthesis from galactose is negligible (28). These observations suggest that incomplete G6P-F6P exchange could also contribute to a reduced G2 enrichment. In support of this, rat hepatocytes provided with glucose labeled with ^{3}H and ^{14}C in position 2 retained ~40% of ^{3}H relative to ^{14}C in glycogen indicating that ~40% of glucose did not undergo G6P-F6P cycling en route to glycogen synthesis (29). This is in good agreement with our estimate of the fraction of direct pathway flux (~37%) that did not experience G6P-F6P exchange. Potential mechanisms that could limit G6P-F6P exchange during fed conditions include the preferential recruitment of G6P derived from glucokinase by the glycogen synthesis pathway (30,31). In contrast, fasting conditions favor more complete exchange of G2 and body water because G6P derived from glycogen is not preferentially converted to glucuronide over G6P derived from gluconeogenesis. Moreover, cycling of glucose and G6P provides additional opportunity for ^{2}H-incorporation by G6P-F6P exchange.

In conclusion, we demonstrate a practical and economical method for quantifying the sources of hepatic glucose and glycogen synthesis in type 1 diabetic patients that can be performed in a standard hospital setting. The analysis reveals significant differences in these parameters between type 1 diabetic patients and healthy subjects and this information may be highly relevant for assessing glycemic control. These data are consistent with previous measurements obtained by clinical ^{13}C MR spectroscopy and glucose tracers. Since it is does not require any specialized clinical equipment or personnel, the ^2H_2O/acetaminophen assay should make these measurements available to a much wider clinical research community.

ACKNOWLEDGMENTS

This work was supported through a Juvenile Diabetes Research Foundation International grant (JDRFI 5-2004-306), the Portuguese Foundation of Science and Technology, and the University of Coimbra Faculty of Medicine Research Support Program.

We acknowledge the insightful comments of the reviewers and excellent technical assistance and support provided by the nursing staff of the Endocrinology Department of University Hospital of Coimbra.

REFERENCES

1. Newgard CB, Hirsch LJ, Foster DW, McGarry JD: Studies on the mechanism by which exogenous glucose is converted into liver-glycogen in the rat: a direct or an indirect pathway. *J Biol Chem* 258:8046–8052, 1983
2. Newgard CB, Hirsch LJ, Foster DW, McGarry JD: Hepatic glycogen-synthesis in the rat: a direct or an indirect pathway. *Fed Proc* 42:2166–2166, 1983
3. Scofield RF, Kosugi K, Schumann WC, Kumaran K, Landau BR: Quantitative estimation of the pathways followed in the conversion to glycogen of glucose administered to the fasted rat. *J Biol Chem* 260:8777–8782, 1985
4. Shulman GI, Cline G, Schumann WC, Chandramouli V, Kumaran K, Landau BR: Quantitative comparison of pathways of hepatic glycogen repletion in fed and fasted humans. *Am J Physiol* 259:E335–E341, 1990
5. Taylor R, Magnusson I, Rothman DL, Cline GW, Caumo A, Cobelli C, Shulman GI: Direct assessment of liver glycogen storage by C-13 nuclear magnetic resonance spectroscopy and regulation of glucose homeostasis after a mixed meal in normal subjects. *J Clin Invest* 97:126–132, 1996
6. Bischof MG, Krssak M, Krebs M, Bernroider E, Stingl H, Waldhausl W, Roden M: Effects of short-term improvement of insulin treatment and glycemia on hepatic glycogen metabolism in type 1 diabetes. *Diabetes* 50:392–398, 2001
7. Hwang JH, Perseghin G, Rothman DL, Cline GW, Magnusson I, Petersen KF, Shulman GI: Impaired net hepatic glycogen synthesis in insulin-dependent diabetic subjects during mixed meal ingestion: a ^{13}C nuclear magnetic resonance spectroscopy study. *J Clin Invest* 95:783–787, 1995
8. Bischof MG, Bernroider E, Krssak M, Krebs M, Stingl H, Nowotny P, Yu CL, Shulman GI, Waldhausl W, Roden M: Hepatic glycogen metabolism in type 1 diabetes after long-term near normoglycemia. *Diabetes* 51:49–54, 2002
9. Petersen KF, Price TB, Bergeron R: Regulation of net hepatic glycogenolysis and gluconeogenesis during exercise: impact of type 1 diabetes. *J Clin Endocrinol Metab* 89:4656–4664, 2004
10. Kunert O, Stingl H, Rosian E, Krssak M, Bernroider E, Seebacher W, Zangger K, Staehr P, Chandramouli V, Landau BR, Nowotny P, Waldhausl W, Haslinger E, Roden M: Measurement of fractional whole-body gluconeogenesis in humans from blood samples using ^{2}H nuclear magnetic resonance spectroscopy. *Diabetes* 52:2475–2482, 2003
11. Rothman DL, Magnusson I, Katz LD, Shulman RG, Shulman GI: Quantitation of hepatic glycogenolysis and gluconeogenesis in fasting humans with ^{13}C NMR. *Science* 254:573–576, 1991
12. Brehm ARW, Krssak M, Anderwald C, Bernroider E, Shulman GI, Cobelli C, Hofer A, Nowotny P, Waldhausl W, Roden M: Hepatic glucose metabolism in Type 2 diabetes after mixed meal ingestion. *Diabetologia* 45: 581, 2002
13. Magnusson I, Rothman DL, Jucker B, Cline GW, Shulman RG, Shulman GI: Liver glycogen turnover in fed and fasted humans. *Am J Physiol* 266:E796–E803, 1994
14. Landau BR, Wahren J, Chandramouli V, Schumann WC, Ekberg K, Kalhan SC: Contributions of gluconeogenesis to glucose production in the fasted state. *J Clin Invest* 98:378–385, 1996

15. Burgess SC, Weis B, Jones JG, Smith E, Merritt ME, Margolis D, Sherry AD, Malloy CR: Noninvasive evaluation of liver metabolism by ^{2}H and ^{13}C NMR isotopomer analysis of human urine. *Anal Biochem* 312:228–234, 2003
16. Ribeiro A, Caldeira MM, Carvalheiro M, Bastos M, Baptista C, Fagulha A, Barros L, Barosa C, Jones JG: Simple measurement of gluconeogenesis by direct ^{2}H NMR analysis of menthol glucuronide enrichment from $^{2}H_2O$. *Magn Res Med* 54:429–434, 2005
17. Jones JG, Barosa C, Gomes F, Mendes AC, Delgado TC, Diogo L, Garcia P, Bastos M, Barros L, Fagulha A, Baptista C, Carvalheiro M, Caldeira MM: NMR derivatives for quantification of ^{2}H and ^{13}C-enrichment of human glucuronide from metabolic tracers. *J Carb Chem* 25:203–217, 2006
18. Jones JG, Merritt M, Malloy CR: Quantifying tracer levels of $^{2}H_2O$ enrichment from microliter amounts of plasma and urine by ^{2}H NMR. *Magn Res Med* 45:156–158, 2001
19. Weis BC, Margolis D, Burgess SC, Merritt ME, Wise H, Sherry AD, Malloy CR: Glucose production pathways by ^{2}H and ^{13}C NMR in patients with HIV-associated lipoatrophy. *Magn Res Med* 51:649–654, 2004
20. Perdigoto R, Furtado AL, Porto A, Rodrigues TB, Geraldes C, Jones JG: Sources of glucose production in cirrhosis by $^{2}H_2O$ ingestion and ^{2}H NMR analysis of plasma glucose. *Biochim Biophys Acta* 1637:156–163, 2003
21. Jones JG, Solomon M: A., Cole SM, Sherry AD, Malloy CR: An integrated ^{2}H and ^{13}C NMR study of gluconeogenesis and TCA cycle flux in humans. *Am J Physiol* 281:E848–E851, 2001
22. Chandramouli V, Ekberg K, Schumann WC, Kalhan SC, Wahren J, Landau BR: Quantifying gluconeogenesis during fasting. *Am J Physiol* 273:E1209–E1215, 1997
23. Schleucher J, Vanderveer P, Markley JL, Sharkey TD: Intramolecular deuterium distributions reveal disequilibrium of chloroplast phosphoglucose isomerase. *Plant Cell Environ* 22:525–533, 1999
24. Leadlay PF, Albery WJ, Knowles JR: Energetics of triosephosphate isomerase: deuterium-isotope effects in enzyme-catalyzed reaction. *Biochemistry* 15:5617–5620, 1976
25. Hellerstein MK, Greenblatt DJ, Munro HN: Glycoconjugates as noninvasive probes of intrahepatic metabolism: 1. Kinetics of label incorporation with evidence of a common precursor UDP-glucose pool for secreted glycoconjugates. *Metab Clin Exp* 36:988–994, 1987
26. Jungermann K, Thurman RG: Hepatocyte heterogeneity in the metabolism of carbohydrates. *Enzyme* 46:33–58, 1992
27. Jungermann K: Functional heterogeneity of periportal and perivenous hepatocytes. *Enzyme* 35:161–180, 1986
28. Stingl H, Chandramouli V, Schumann WC, Brehm A, Nowotny P, Waldhausl W, Landau BR, Roden M: Changes in hepatic glycogen cycling during a glucose load in healthy humans. *Diabetologia* 49:360–368, 2006
29. Katz J, Wals PA, Rognstad R: Glucose phosphorylation, glucose-6-phosphatase, and recycling in rat hepatocytes. *J Biol Chem* 253:4530–4536, 1978
30. Agius L, Peak M: Intracellular binding of glucokinase in hepatocytes and translocation by glucose, fructose and insulin. *Biochem J* 296:785–796, 1993
31. Agius L: The physiological role of glucokinase binding and translocation in hepatocytes. In *Advances in Enzyme Regulation*. Vol. 38. 1998, p. 303–331