Estimation of glucose carbon recycling in children with glycogen storage disease: A $^{13}$C NMR study using [U-$^{13}$C]glucose

($^{13}$C]glucose C-1–C-2 coupling/gluconeogenesis/mechanism for glucose production)

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ABSTRACT A stable isotope procedure to estimate hepatic glucose carbon recycling and thereby elucidate the mechanism by which glucose is produced in patients lacking glucose 6-phosphatase is described. A total of 10 studies was performed in children with glycogen storage disease type I (GSD-I) and type III (GSD-III) and control subjects. A primed dose-constant nasogastric infusion of D-[U-$^{13}$C]glucose (>99% $^{13}$C-enriched) or an infusion diluted with unlabeled glucose solution was administered following different periods of fasting. Hepatic glucose carbon recycling was estimated from $^{13}$C NMR spectra. The recycling parameters were derived from plasma $\beta$-glucose C-1 splitting pattern, doublet/singlet values of plasma glucose C-1 in comparison to doublet/singlet values of known mixtures of [U-$^{13}$C]glucose and unlabeled glucose as a function of $^{13}$C enrichment of glucose C-1. The fractional glucose C-1 enrichment of plasma glucose samples was analyzed by $^1$H NMR spectroscopy and confirmed by gas chromatography/mass spectrometry. The values obtained for GSD-I patients coincided with the standard [U-$^{13}$C]glucose dilution curve. These results indicate that the plasma glucose of GSD-I subjects comprises only a mixture of 99% $^{13}$C-enriched D-[U-$^{13}$C]glucose and unlabeled glucose but lacks any recycled glucose. Significantly different glucose carbon recycling values were obtained for two GSD-III patients in comparison to GSD-I patients. Our results eliminate a mechanism for glucose production in GSD-I children involving gluconeogenesis. However, glucose release by amylo-1,6-glucosidase activity would result in endogenous glucose production of non-$^{13}$C-labeled and nonrecycled glucose carbon, as was found in this study. In GSD-III patients gluconeogenesis is suggested as the major route for endogenous glucose synthesis. The contribution of the triose-phosphate pathway in these patients has been determined. The significant difference of the glucose C-1 splitting pattern in plasma GSD-III and control subjects, in comparison to GSD-I plasma, can be used as a parameter for estimating glucose recycling. This approach can be developed as a noninvasive diagnostic test for inborn enzymatic defects involving gluconeogenesis.

Following prolonged fasting, when glycogen stores are depleted, gluconeogenesis from three carbon intermediates is the major route for glucose production (1). The release of glucose from the cell produced either by glycogenolysis or gluconeogenesis involves the hydrolisis of glucose 6-phosphate by glucose 6-phosphatase.

As a result of impairment of glucose 6-phosphatase activity, children with glycogen storage disease type I (GSD-I) are susceptible to hypoglycemia during short periods of fasting (2). However, a limited amount of endogenous glucose production was found in these children (3–5). By using tracer methodology, we have recently shown that the source of endogenous glucose production is not from gluconeogenesis (5, 6), in contrast to patients with amylo-1,6-glucosidase deficiency (GSD-III), whose endogenous glucose is mainly derived from gluconeogenic precursors (6).

An understanding of inherited disorders of carbohydrate metabolism should clarify our knowledge of normal metabolism and define biochemical adaptations available to the human organism. A noninvasive and nonradioactive approach was undertaken to assess the mechanism by which glucose is produced in children with GSD-I whose glucose 6-phosphatase activity is impaired. D-[U-$^{13}$C]Glucose was administered to GSD-I patients and glucose carbon recycling was determined by monitoring $^{13}$C NMR resonances of plasma glucose at position C-1 coupled to C-2. Endogenous dilution by unlabeled glucose molecules in comparison to dilution by recycled glucose molecules enabled us to suggest mechanisms for glucose production in two types of GSD patients, type I and type III.

The ultimate goal of this work is to develop safe noninvasive and nonradioactive diagnostic tests for patients suspected of GSD and other enzymatic disorders involving gluconeogenesis.

EXPERIMENTAL PROCEDURES

Subjects and Study Design. The study included four GSD-I patients. One patient was studied three times following different glucose infusion rates and different mixtures of D-[U-$^{13}$C]glucose and unlabeled glucose. Two GSD-III subjects were studied following overnight fasting (9–12 hr). The control group was chosen for this study because they temporarily required supplementary nasogastric feeding due to feeding difficulties. A total of 10 studies was performed in GSD and control subjects, each of whom received a primed dose-constant nasogastric infusion of D-[U-$^{13}$C]glucose (99% $^{13}$C-enriched) or an infusion diluted with unlabeled glucose solution. The primed dose was 25% of the fractional [U-$^{13}$C]glucose in the infusion mixture. Glucose was infused at a range of 0.4–6 mg/kg per min; blood samples were taken at intervals of up to 100 min to determine steady-state conditions. The diagnosis in each patient (aged 6–64 months) was based on the characteristic clinical presentation and the enzymatic confirmation of the metabolic defect in hepatic tissue (glucose 6-phosphatase activity in GSD-I) and in peripheral blood leukocytes (amylo-1,6-glucosidase activity in GSD-III). All GSD-I patients had growth retardation prior to initiation of carbohydrate feeding. The study received the approval of the Human Studies Committee of the Hadassah Medical Center, Jerusalem. Informed consent was obtained from the parents of the children.

Abbreviations: GSD, glycogen storage disease; d/s, doublet/singlet.

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**Materials.** D-[U-13C]Glucose (99% enriched) was prepared in our laboratory from algae grown on 13CO2 (>99% enriched) (7).

**NMR Spectroscopy.** High-resolution 13C NMR spectra were obtained with a Bruker 500-MHz spectrometer operating at 125.76 MHz with composite pulse decoupling to reduce effects from dielectric heating and to maintain the sample temperature at about 20°C. Spectra were obtained by using a 5-mm 13C NMR probe with the following parameters: a 60° pulse angle with a 2-s repetition time, a 26.5-KHz spectral width, and a 32 K block. Field stabilization was accomplished by locking on 6% 2H2O.

**Determination of Isotopic Enrichments of [U-13C]Glucose at Position C-1.** Two approaches were used to determine 13C enrichments of [U-13C]glucose at position C-1 in deproteinized plasma and in infused glucose solutions.

(i) **GC/MS measurements.** Glucose was derivatized to 1,2,3,4,6-penta-O-trimethylsilyl α- and β-d-glucose anomers, using Supelco silyl reagent Sylon BFT [bis(trimethylsilyl)trifluoroacetamide/chlorotrimethylsilane, 99:1] in pyridine solution 1:1 (8). Two main ions are obtained by chemical ionization GC/MS: m/z 361 (MH – 180) and m/z 191, corresponding to an intact glucose molecule and to a C-1 glucose fragment, respectively. The ions m/z 191 and 192 were used in this study for measuring glucose C-1 fractional 13C enrichment. GC/MS conditions for glucose and alanine separation and their 13C enrichments were performed as described (9, 10).

(ii) **1H NMR measurements.** The 13C enrichment of α-d-glucose C-1 was measured from the 1H NMR spectrum at 500 MHz in a standard 5-mm 1H NMR probe with a Bruker 500-MHz spectrometer. A 40° pulse angle (6 μs) with a 10-s relaxation delay was used. During the relaxation delay, the water resonance was saturated with a single radio frequency field. Spectra were obtained with a sweep width of 6000 KHz. Samples were heated to 330 K to shift the H2O resonance upfield from the C-1 proton resonance of α-d-glucose.

**RESULTS AND DISCUSSION**

When D-[U-13C]glucose is administered, it is metabolized to three carbon intermediates, which are diluted by the endogenous nonlabeled pool. Further dilution of the three carbon compounds may occur as a result of interaction of the three carbon compounds with tricarboxylic acid cycle intermediates. Thus, the recycled glucose molecules synthesized via the triose-phosphate pathway are no longer uniformly 13C labeled, as shown in Fig. 1.

Although the generation of free glucose from glucose 6-phosphate is impaired in GSD-I patients, it has recently been shown that a limited amount of glucose is produced in these patients (3-5), but the mechanism by which glucose is synthesized in GSD-I patients is still questionable. A procedure to estimate hepatic glucose carbon recycling and thereby elucidate the mechanism by which glucose is produced in patients lacking glucose 6-phosphatase activity is presented in this paper.

**Analysis of [U-13C]Glucose at Position C-1 of Nonrecycled Glucose Solutions.** The 13C-13C splitting pattern at a specific site gives the relative amount of 13C label of two adjacent carbon atoms (11–15). Fig. 2 represents 13C NMR spectra from β-glucose C-1 of several mixtures of 99% enriched D-[U-13C]glucose (1–20%) with unlabeled glucose. The 13C NMR resonance of the β anomer of glucose C-1 is well resolved and the splitting pattern, 1J_{C-C} (46 Hz), arises only from one adjacent glucose carbon at position 2. Although α and β anomers of glucose reveal similar splitting patterns, the β anomer is preferable for this analysis since its intensity is about 40% higher than the corresponding α anomer. As seen in Fig. 2, the center peak arises from the nonenriched glucose in the mixture (it corresponds to the natural abundance carbon-13) (1.1%), whereas the doublet resonances of [U-13C]glucose arise from glucose C-1–C-2 coupling. The center peaks decrease, whereas the outer peaks increase with the increase of [U-13C]glucose fraction in the mixture. It is worth noting that doublet/singlet (d/s) derived from a mixture as low as 1% D-[U-13C]glucose could be determined (Fig. 2, spectrum A). Spectrum Z is of the highly enriched D-[U-13C]glucose (99%); only a residual trace of the singlet peak can be seen, since the compound contains <1% 12C glucose. Spectra A–Z reveal several peaks that are the superposition of all isotopomers present. These peaks arise from 12 coupling of glucose C-1 with C-3 and C-5. This enables one to monitor 13C-13C coupling of nonadjacent carbon atoms; thus, changes of 13C enrichment of glucose carbons at positions C-2, C-3, and C-5 coupled to glucose C-1 can be measured from the glucose C-1 resonance splitting pattern.

![Fig. 1. Schematic presentation of the labeling pattern of plasma glucose and three carbon molecule pools following D-[U-13C]glucose administration. OAA, oxalacetate; TCA, tricarboxylic acid; PEP, phosphoenolpyruvate.](image-url)
Linear regression analysis of β-glucose C-1 d/s peak areas as a function of 13C enrichment of glucose C-1 is presented in Fig. 3A. Fig. 3A represents a dilution curve of [U-13C]glucose by unlabeled glucose molecules. The 13C fractional enrichment of glucose at position C-1 of each glucose sample was measured from 1H NMR spectra of α-glucose (Fig. 4) and/or by GC/MS analysis of the glucose C-1 fragment.

13C NMR Measurements of Plasma Derived from GSD-I and GSD-III and Control Subjects. At 100–120 min after nasogastric infusion of D-[U-13C]glucose into all subjects when steady-state conditions had been established, plasma samples were drawn for 13C NMR measurements. Spectra shown in Fig. 5 are those of GSD-I patients (A), a GSD-III patient (B), and a control subject (C). The intense 13C resonances of glucose arise from the infused glucose, glucose produced following the infusion of the labeled glucose, and the endogenous glucose pool. All carbon resonances having multiplet structure are due to 13C-13C coupling either with one or two adjacent carbon-13 atoms. The resolved β-glucose C-1 multiple resonances of plasma spectra enabled us to measure the fractional doublet peak area. The d/s ratios of β-glucose C-1 resonances derived from GSD-I and GSD-III patients and control subjects (10 studies) are summarized in Table 1. Extended views of β-glucose C-1 and lactate C-3 resonances derived from two GSD-I plasma samples are presented in Fig. 5A, spectrum a, subject 3 (GSD-I), and spectrum b, subject 1 (GSD-I). The different d/s ratios of β-glucose C-1 resonances shown for spectra a and b are a result of different plasma 13C glucose enrichments [8.5% and 3.0% (atom%) 13C excess, respectively], since different rates of glucose infusion and different fractions of [U-13C]glucose in the infused mixtures were used (11% and 9.3% 13C-enriched glucose for

![Graph](image-url)

**Fig. 3.** (A) Linear regression analysis of proton decoupled 13C NMR of β-glucose C-1 fraction (see Fig. 2) d/s ratio with respect to their glucose C-1 enrichments. Glucose samples are mixtures of D-[U-13C]glucose (>99% enriched) with unlabeled glucose. R = 0.992; Y = −0.035 + 0.68X. (B) GSD-I plasma glucose C-1 d/s as a function of glucose C-1 13C enrichment (●) values coincide with the standard dilution curve (—) shown in A. GSD-III patients (7 and 8) and control subjects (9 and 10) deviate from the standard dilution curve (—).

![Graph](image-url)

**Fig. 4.** Representative 1H NMR spectra (500 MHz) of the α-glucose C-1 fraction of infused glucose and of plasma glucose of a GSD-I patient. The percentage of 13C labeling of glucose C-1 was calculated from the ratio of 13C satellite peak areas with respect to the total peak areas of 12C and 13C satellite signals: \( \frac{2(13C)/(12C) + 2(13C)}{100} \).
natural abundance of $^{13}$C (1.1%); in spectrum b the singlet area and the doublet area are similar; thus, the lactate is $\approx 1\%$ $^{13}$C-enriched. It is about 2.5 times more diluted than plasma glucose in this patient. The $^{13}$C enrichment of alanine and/or lactate has been confirmed by MS and was found to be 1.3% for GSD-I (spectrum b) and 4% for GSD-I (spectrum a). The observation of $[^{13}$C]$\alpha$-lactate in plasma of GSD-I patients is in accordance with our recent GC/MS results of $[^{13}$C]$\alpha$-alanine found in the plasma of GSD-I patients. We have found that the ratio of $[^{13}$C]$\alpha$-lactate to $[^{13}$C]$\alpha$-alanine (a lactate of plasma derived from GSD-I patients is even more responsive to glucose loads than glucose production rates (6). The $^{13}$C NMR spectrum derived from the plasma of a GSD-III patient (subject 8 in Table 1) is a result of highly enriched glucose (15% enriched) and $\approx 4\%$ enriched lactate. The spectrum also reveals resonances at the region 20–80 ppm corresponding to the CH$_3$ and CH$_2$ groups of keto bodies such as $\beta$-hydroxybutyrate. Most of these carbon resonances appear as singlets, indicating $<1\%$ carbon enrichment. Since these molecules are derived from acetyl-CoA carbons, this proves that following [U-$^{13}$C]glucose infusion acetyl-CoA carbons are not $^{13}$C-enriched.

A representative spectrum of plasma glucose separated from salt and acids is shown in the expanded view of the 96–98 ppm region (Fig. 5A, spectrum c). The $^{13}$C resonances are well resolved, as shown for the mixed [U-$^{13}$C]glucose solutions in Fig. 2. However, for the determination of the d/s ratio of $^{13}$C, purification of glucose can be omitted; therefore, most measurements were done on deproteinized plasma samples only.

The $^{13}$C fractional enrichment of each C-1 glucose sample was determined from its $^1$H NMR spectrum and confirmed by GC/MS of glucose C-1. $^1$H NMR spectra of $\alpha$-glucose $^{13}$C labeled at the C-1 position of infused and plasma samples are shown in Fig. 4. The d/s peak areas derived from the $^{13}$C NMR plasma glucose C-1 splitting pattern of each GSD-I patient are depicted as a function of their $^{13}$C enrichments in Fig. 3B. The glucose recycling factor (see Table 1) is defined as the ratio of (d/s)$_h$ to the measured plasma to (d/s)$_c$ of the standard curve at the same $^{13}$C enrichment found for the plasma glucose: $GR = [1 - (D/S)_{pa}/(D/S)_{sc}] \times 100$.

The values obtained for GSD-I patients coincided with the standard [U-$^{13}$C]glucose dilution curve. These results indicate that the plasma glucose of GSD-I subjects comprises only a mixture of 99% $^{13}$C-enriched n-[U-$^{13}$C]glucose and unlabeled glucose but lacks any recycled glucose (Fig. 1).

Since a few of the GSD-I patients could not tolerate fasting (>4 hr) without developing hypoglycemia, glucose was in-
fused at a rate of 2.7–6.1 mg/kg per min. As shown in Table 1, under these conditions endogenous glucose production is depressed as glucose levels are increased (3, 6). However, no significant differences in glucose carbon recycling (see Table 1) could be noted among the GSD-I children, even when glucose infusion rates were sufficiently low (2.7 and 3.0 mg/kg per min) to induce maximal glucose production. Under these infusion conditions the endogenous glucose production fraction \( (R_p) \) of the total glucose appearance \( (R_a) \) reached \( \approx 50\% \) (subjects 3 and 5 in Table 1). \( R_p \) and \( R_a \) values were calculated from plasma glucose \( ^{13}\text{C} \) enrichments and the glucose infusion rates \( (r) \) (5, 16). Results are presented from similar glucose infusion rates, but different mixtures of labeled and unlabeled glucose were used to provide study feasibility at low enrichments of \( ^{13}\text{C} \)-labeled plasma metabolites.

Significantly different glucose carbon recycling values were obtained for two GSD-III patients in comparison to GSD-I patients. The same procedure of \( ^{13}\text{C} \) and \( ^{1}\text{H} \) NMR plasma measurements was carried out. Since these children could tolerate longer periods of fasting, only the labeled [U-\( ^{13}\text{C} \)]glucose was administered following overnight fasting (9–12 hr) with lower glucose infusion rates (0.4–0.6 mg/kg per min).

As seen in Fig. 3B, d/s as a function of \( ^{13}\text{C} \) enrichments of glucose C-1, obtained from the two GSD-III patients, demonstrates a greatly reduced degree of glucose C-1–C-2 coupling. The singlet resonance of glucose C-1 no longer arises only from the endogenous nonlabeled glucose. Since the center peak is a singlet resonance of glucose labeled at position C-1, but not at position C-2, we assume that a great portion of glucose molecules derived from the plasma of GSD-III patients does not have C-1 and C-2 intact; therefore, the ratio of d/s is decreased significantly, 29% and 33%, respectively, from the standard curve of [U-\( ^{13}\text{C} \)]glucose d/s (Fig. 3A) at the same glucose C-1 enrichment. Thus, the values for GSD-I patients, whose d/s ratios coincide with the standard curve, are significantly different. Two control infants were studied; their plasma glucose d/s values also revealed significant deviation from the [U-\( ^{13}\text{C} \)]glucose standard curve, 10% and 17% in comparison to zero deviation found for all GSD-I patients.

A different approach for determination of glucose recycling in GSD-I and GSD-III patients has been recently presented using [U-\( ^{13}\text{C} \)]glucose and GC/MS (5, 6). The results presented in these studies demonstrate that in GSD-I patients, the available three-carbon molecule pool is \( ^{13}\text{C} \)-enriched and also recycled, and plasma glucose carbons are not recycled, whereas GSD-III glucose carbons and three-carbon molecules are recycled. We conclude that glucose in GSD-I patients is not produced from the triose-phosphate pathway, and during infusion of labeled glucose it is produced from unlabeled sources.

It has been proposed (3) that hepatic glucose is produced in GSD-I children almost exclusively from gluconeogenesis, and the residual activity of glucose 6-phosphatase is responsible for the release of glucose from glucose 6-phosphate. This mechanism does not agree either with the result presented in this study or with our recent results measuring the isotopomer population of \( [^{13}\text{C}] \)glucose by GC/MS (5, 6). The recently reported complete suppression of glucose production using high rates of glucose infusion (4) makes it unlikely that glucose production was due to the activity of \( \alpha-1,4\text{-gluicosidase (acid maltase)}, \) since this enzyme would not be expected to be responsive to changes in substrate and hormone concentrations.

Our results eliminate a mechanism for glucose production in GSD-I children involving gluconeogenesis. However, glucose release by amylo-1,6-glucosidase activity would result in endogenous production of non-\( ^{13}\text{C} \)-labeled and nonrecycled glucose carbon, as was found in this study. In GSD-III, glucose released from glycogen is limited. Following an overnight fast, the impairment of amylo-1,6-glucosidase activity enhances glucose production by gluconeogenesis. Indeed, extensive glucose recycling was measured in these patients. The elevated gluconeogenesis may be an adaptive mechanism in GSD-III patients to protect the fasting patient from hypoglycemia.

In summary, an approach for measuring hepatic glucose recycling, in children with GSD-I and GSD-III, by using [U-\( ^{13}\text{C} \)]glucose and \( ^{13}\text{C} \) NMR, was carried out in this study by measuring plasma glucose \( ^{13}\text{C} \)-\( ^{13}\text{C} \) coupling of two adjacent glucose carbons C-1–C-2. Only residual hepatic activity of glucose 6-phosphatase in GSD-I would result in newly synthesized recycled glucose. Our findings reveal that glucose production in these patients is nonrecycled; thus, the proposed mechanism based on residual glucose 6-phosphatase activity can be eliminated. In contrast, in GSD-III patients gluconeogenesis is suggested as the major route for endogenous glucose synthesis. The contribution of the triose-phosphate pathway in GSD-III patients was determined. The significant difference of the glucose C-1 splitting pattern of GSD-III plasma and control subjects, in comparison to GSD-I plasma, can be used as a parameter for estimating glucose recycling. This approach can be developed as a noninvasive diagnostic test for inborn enzymatic defects involving gluconeogenesis.

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