Comparison of [3,4-$^{13}C_2$]Glucose to [6,6-$^{2}H_2$]Glucose as a Tracer for Glucose Turnover by Nuclear Magnetic Resonance

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A recently introduced tracer, [3,4-$^{13}C_2$]glucose, was compared to the widely used tracer, [6,6-$^{2}H_2$]glucose, for measurement of whole-body glucose turnover. The rate of glucose production (GP) was measured in rats after primed infusions of [3,4-$^{13}C_2$]glucose, [6,6-$^{2}H_2$]glucose, or both tracers simultaneously followed by a constant infusion of tracer(s) over 90 min. Blood glucose was purified and converted into monoacetone glucose for analysis by ^{13}C NMR (for [3,4-$^{13}C_2$]glucose) or ^{1}H and ^{2}H NMR (for [6,6-$^{2}H_2$]glucose). The values of GP measured during infusion of each single tracer were not significantly different. In rats infused with both tracers simultaneously, GP was identical as reported by each tracer, 42 ± 4 μmol/kg/min. Since ^{2}H and ^{13}C enrichment in glucose is typically much less than 2% for in vivo studies, [3,4-$^{13}C_2$]glucose does not interfere with measurements of ^{13}C or ^{2}H enrichment patterns and therefore is valuable when multiple metabolic pathways are being evaluated simultaneously. Magn Reson Med 53:1479–1483, 2005. © 2005 Wiley-Liss, Inc.

Key words: stable isotope tracers; ^{13}C; ^{2}H; NMR; glucose turnover

Multiple tracer methods have been introduced to measure systemic glucose turnover in vivo. These methods share the same physiologic principles, but differ in technical details such as the site of isotope labeling, the use of stable or radioactive isotopes, and detection techniques. Stable isotopes are now preferred for practical and ethical reasons despite lower sensitivity compared to radiotracers. If glucose turnover is the sole purpose of an experiment, mass spectrometry is usually chosen because of high sensitivity, simplicity of data interpretation, and wide application (1,2). However, if multiple stable isotopes or labeling patterns are used in a single study to probe additional metabolic pathways, then NMR offers the benefit that multiple sites of ^{13}C or ^{2}H labeling in a glucose molecule are easily distinguished. This property offers an overwhelming advantage compared to mass spectrometry.

There is increasing interest in simultaneous measurement of both glucose turnover and other metabolic fluxes in vivo. For example, administration of $^{2}H_2O$ followed by measurement of deuterium enrichment in positions 2, 5, and 6 of glucose by mass spectrometry allowed Saadatian et al. to calculate the relative rates of glucose production (GP) from glycerol, glycogen, and the TCA cycle (3). After co-administration of $^{2}H_2O$ (oral) plus [6,6-$^{2}H_2$]glucose by continuous i.v. infusion, plasma glucose becomes enriched in multiple sites. The ratio of enrichment in position 5 compared to position 2 (or plasma water) yielded the fraction of plasma glucose derived from gluconeogenesis; whole-body turnover of glucose was measured from the ^{2}H enrichment at position 6. However, if detected by mass spectrometry, even [6,6-$^{2}H_2$]glucose is not an adequate tracer if other pathways are probed simultaneously by $^{2}H_2O$ and by ^{13}C-enriched tracers (since $m + 2$ could be due to additional neutrons from either ^{2}H or ^{13}C enrichment or both).

A recently introduced tracer, [3,4-$^{13}C_2$]glucose, has multiple advantages compared to other ^{2}H- or ^{13}C-labeled glucoses (4). Unlike glucose enriched with a hydrogen tracer in position 2, the ^{13}C labeling is not influenced by cycling between glucose-6-phosphate and fructose-6-phosphate. This tracer also offers major advantages when detected by NMR. Glucose itself is an unfavorable molecule for NMR detection because of poor chemical shift dispersion and the presence of anomers, which effectively reduce signal and further complicate the spectra. A simple derivative, monoacetone glucose (1,2-diisopropylidene glucofuranose, also termed MAG), offers well-resolved ^{2}H and ^{13}C NMR spectra while preserving the informative ^{2}H and ^{13}C distribution. Unlike [1,6-$^{13}C_2$]MAG, [3,4-$^{13}C_2$]MAG yields a characteristic pair of doublets in a ^{13}C NMR spectrum due to spin–spin coupling, which is easily observed. Most importantly, detection with NMR has the considerable advantage that the presence of ^{13}C does not interfere with measurement of ^{2}H enrichment by ^{2}H NMR at typical levels of enrichment.

The goal of this study was to compare GP measured by a standard tracer, [6,6-$^{2}H_2$]glucose, to GP measured by NMR detection of [3,4-$^{13}C_2$]glucose. Also, a convenient method to quantify ^{2}H enrichment in plasma glucose is introduced using an internal reference, deuterated dimethylformamide (DMF-d_7). The rates of GP were measured in rats receiving primed infusions of [3,4-$^{13}C_2$]glucose or [6,6-$^{2}H_2$]glucose or both tracers simultaneously. After infusion, blood glucose was converted to MAG and tracer enrich-

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Grant sponsor: National Science Foundation; Grant sponsor: National Institutes of Health; Grant numbers: RR-02584, DK16194, and HL-34557.

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Received 18 August 2004; revised 8 December 2004; accepted 12 January 2005.

DOI 10.1002/mrm.20496
Published online in Wiley InterScience (www.interscience.wiley.com).

ments in blood glucose were obtained from the ^{13}C-NMR spectrum of MAG for [3,4-$^{13}C_2$]glucose and both ^{1}H- and ^{2}H-NMR spectra of MAG for [6,6-$^2H_2$]glucose using internal references. The convenience of probing multiple pathways in a single study coupled with the inherent safety of stable isotopes will make NMR spectroscopy the preferred method for analysis of glucose synthesis and kinetics in patients.

METHODS

Materials

[3,4 -$^{13}C_2$]Glucose (99%) was purchased from Omicron Biochemicals (South Bend, IN, USA). Formic acid-d_2 (98%) was obtained from Cambridge Iostopes (Andover, MA, U.S.A.). [6,6-$^2H_2$]Glucose (98%) was obtained from Isotec (Miamisburg, OH, USA). DMF-d_7 (99.5%), natural abundance *N*,*N*-dimethylformamide (DMF), deuterated acetonitrile (99.8%), and other common chemicals were purchased from Aldrich (Milwaukee, WI, USA).

Protocol

This study was approved by the Institutional Animal Care and Use Committee at UT Southwestern Medical Center. Male Sprague–Dawley rats weighing 201 ± 12 g were fasted for 24 h with free access to water, after which the jugular vein was cannulated under ketamine/xylazine anesthesia. Rats received primed infusions of [3,4-$^{13}C_2$]glucose (n = 6), [6,6-$^2H_2$]glucose (n = 6), or a mixture of both tracers (n = 6). In each case, 35 μmol/kg bolus of each tracer was given and followed by continuous infusion at the rate of 1 μmol/kg/min for 90 min. Thus, the glucose amount in both prime and infusion was doubled in rats that received both tracers compared to rats that received a single tracer. At the end of each infusion protocol, ~6 mL of blood was drawn from the descending aorta under general anesthesia and the animal was sacrificed.

Sample Processing

Plasma glucose was assayed enzymatically (5). Whole blood was immediately deproteinized by treatment with cold perchloric acid to the final concentration of 7% by volume. After neutralization with KOH and centrifugation, the supernatant was lyophilized. Dried glucose was converted into MAG (6). MAG samples (varying in ^{2}H enrichment from 0.49 to 9.83%, constant 22 μmol in each sample) for generation of standard curves were prepared by mixing known amounts of natural abundance glucose and [6,6-$^2H_2$]glucose prior to chemical conversion to MAG. For ^{1}H and ^{2}H NMR acquisition, MAG was dissolved either in a mixture of 170 μL acetonitrile, 4 μL water, and 2 μL DMF-d_7 (1.2%) or in a mixture of 164 μL acetonitrile, 6 μL water, and 10 μL deuterated formate (4.2%). After ^{1}H and ^{2}H NMR acquisition, MAG was lyophilized and resuspended in 180 μL deuterated acetonitrile (99.8%) for ^{13}C NMR acquisition.

NMR Spectroscopy

All NMR spectra were collected using a Varian Inova 14.1-T spectrometer (Varian Instruments, Palo Alto, CA, USA) equipped with a 3-mm broadband probe with the observe coil tuned to ^{1}H (600 MHz), ^{2}H (92 MHz), or ^{13}C (150 MHz). After shimming performed on selected ^{1}H resonances of MAG, ^{1}H NMR spectra were acquired using a 90° pulse (11 μs), 8000 Hz sweep width, 47,998 data points, a 3-s acquisition time, and a 1-s interpulse delay at 50°C. ^{1}H NMR spectra were averaged over 16 scans requiring ~2 min. The acetonitrile signal was presaturated using a frequency-selective pulse in acquiring ^{1}H NMR. Proton-decoupled ^{2}H NMR spectra were acquired using a 90° pulse (12.5 μs), 920 Hz sweep width, 1836 number of data points, and a 1-s acquisition time with no further delay at 50°C. Typically 6400–20000 scans were averaged, requiring ~2–5 h. Proton decoupling was performed using a standard WALTZ-16 pulse sequence. ^{13}C NMR spectra were collected using 52° pulse (6.06 μs), 20330 Hz sweep, with 60992 data points, and a 1.5-s acquisition time with no further delay at 25°C (4). Typically 4000–40000 scans were averaged, requiring ~2–18 h. NMR spectra were analyzed using the curve-fitting routine supplied with the NUTS PC-based NMR spectral analysis program (Acorn NMR, Inc., Freemont, CA, USA).

Measurement of Glucose Production

Glucose turnover was measured by dilution of infused [3,4-$^{13}C_2$]glucose or [6,6-$^2H_2$]glucose by endogenous glucose at the end of a constant period of tracer infusion. Excess enrichment of [3,4-$^{13}C_2$]glucose in plasma was measured by ^{13}C NMR spectroscopy as previously described (4), while excess enrichment of [6,6-$^2H_2$]glucose was measured by a combination of ^{1}H and ^{2}H NMR spectroscopy using either deuterated formate, as described previously (7), or DMF-d_7 (1.2%) as an internal reference. Typical ^{1}H and ^{2}H NMR spectra of MAG derived from blood glucose /DMF-d_7 mixture are shown in Fig. 1. One of the methyl resonances of DMF (2.8 ppm) was chosen for use as an internal reference to standardize the measurement of glucose H6 multiplet areas in the ^{1}H ($A_{H6(R+S)}$) and ^{2}H NMR ($A_{D6(R+S)}$) resonances. The DMF methyl resonance at 2.8 ppm was set arbitrarily to 100 in each spectrum. A standard curve was generated using seven samples by plotting actual enrichment of [6,6-$^2H_2$]glucose versus the ratio, $(A_{D6(R+S)})/(A_{H6(R+S)})$ (Fig. 2). The linear correlation shown in Fig. 2 is described by $L = 0.72x + 0.2692$, where L is the % [6,6-$^2H_2$]glucose in each sample and x is the area ratio, $A_{D6(R+S)}/A_{H6(R+S)}$ ($r^2 = 0.996$).

In measuring the excess enrichments of both tracers, standard curves were generated by running known standard samples using the NMR conditions described above. Under these NMR conditions, many signals were not fully relaxed. For example, a 1-s interpulse delay after 90° pulse width in ^{1}H NMR is insufficient for full relaxation. However, data from the standards were collected under identical conditions, so the standard curves remain valid.

GP was calculated from the known infusion rate (R_i), the fraction of infusate glucose that was [6,6-$^2H_2$]glucose or [3,4-$^{13}C_2$]glucose (L_i), and the fraction of plasma glucose that was [6,6-$^2H_2$]glucose or [3,4-$^{13}C_2$]glucose (L_p): $GP = R_i \times (L_i - L_p)/L_p$.

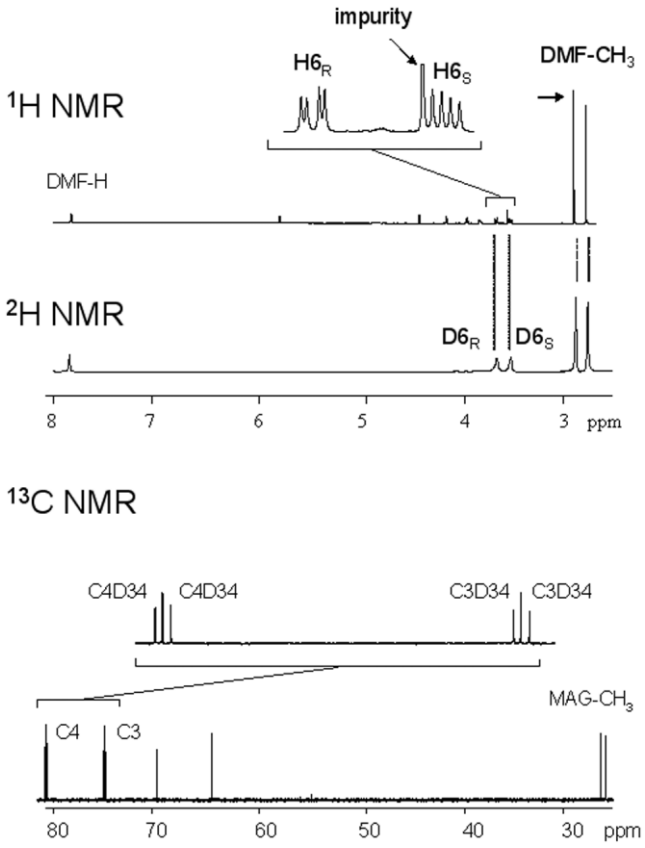

FIG. 1. ^{1}H, proton decoupled ^{2}H, and proton decoupled ^{13}C NMR spectra of MAG derived from plasma glucose of rats infused with [6,6-$^2H_2$]glucose and [3,4-$^{13}C_2$]glucose simultaneously. The ^{1}H NMR spectrum (top) illustrates the methyl peak of DMF-d_7 at 2.8 ppm (indicated by a horizontal arrow), which was used as an internal reference in both the ^{1}H and the ^{2}H spectra. The inset shows the H6 resonances of the ^{1}H NMR spectrum. The ^{2}H NMR spectrum (middle) illustrates a typical spectrum of [6,6-$^2H_2$] MAG plus the ^{2}H resonances of DMF. The ^{13}C NMR spectrum (bottom) shows the characteristic doublets due to $J_{3,4}$ from the intact tracer, [3,4-$^{13}C_2$]glucose. The other resonances at 82, 75, 69, and 65 ppm are the natural abundance signals from carbons 4, 3, 5, and 6, respectively, of MAG.

Statistical Analysis

The data are expressed as means ± SD. Comparisons between groups were performed using one-way analysis of variance. Differences in mean values were considered statistically significant at the probability level of less than 5% ($P < 0.05$).

RESULTS

The three resonances of DMF-d_7 (2.6, 2.8, and 7.95 ppm) are well resolved from all MAG resonances in the ^{1}H and ^{2}H NMR spectra (Fig. 1, upper panels). The methyl resonance at 2.8 ppm was used as a reference because the one at 2.6 ppm was closer to a broad water resonance at ~2.3 ppm, especially in the ^{1}H NMR spectrum (not shown). There was an excellent linear correlation (Fig. 2) between excess enrichment of [6,6-$^2H_2$]glucose versus the ratio, $(A_{D6(R+S)})/(A_{H6(R+S)})$. The doublets of [3,4-$^{13}C_2$]glucose and methyl groups of MAG were also well resolved in the ^{13}C NMR spectrum (Fig. 1, bottom panel) making the excess enrichment of this tracer also easy to evaluate (4). Table 1 summarizes the results from two groups of rats that received either [3,4-$^{13}C_2$]glucose or [6,6-$^2H_2$]glucose alone. Excess enrichment of [6,6-$^2H_2$]glucose (1.87 ± 0.14%) and [3,4-$^{13}C_2$]glucose (2.04 ± 0.04%) did not differ at the end of 90 min but the difference in GP as measured by the two tracers approached statistical significance (50.8 ± 3.9 μmol/kg/min for [6,6-$^2H_2$]glucose versus 45.4 ± 4.7 μmol/kg/min for [3,4-$^{13}C_2$]glucose, $P = 0.058$). In this first set of experiments, formate was used as an internal reference to quantify [6,6-$^2H_2$]glucose excess enrichment (7) while the internal methyl resonances of MAG were used to quantify [3,4-$^{13}C_2$]glucose excess enrichment (4). Plasma glucose in rats that received [6,6-$^2H_2$]glucose tended to be higher (7.1 ± 1.8 versus 6.2 ± 0.8 mM, Table 1) although this trend did not reach statistical significance.

To test whether the trend toward difference in GP as reported by the two tracers might be due to error propagation from (a) two different groups of animals and(b) two different NMR nuclei and samples, GP was measured again in a single group of rats after administration of both tracers simultaneously. This approach takes advantage of the fact that NMR detection of [3,4-$^{13}C_2$]glucose does not interfere with detection of [6,6-$^2H_2$]glucose. In this later group, DMF-d_7 (as described under Methods) was used as an internal reference to quantify [6,6-$^2H_2$]glucose excess enrichment while, just as before, the internal methyl resonances of MAG were used to quantify [3,4-$^{13}C_2$]glucose excess enrichment. As summarized in Table 2, the second set of experiments gave excellent agreement between both tracer enrichment levels and in measures of GP.

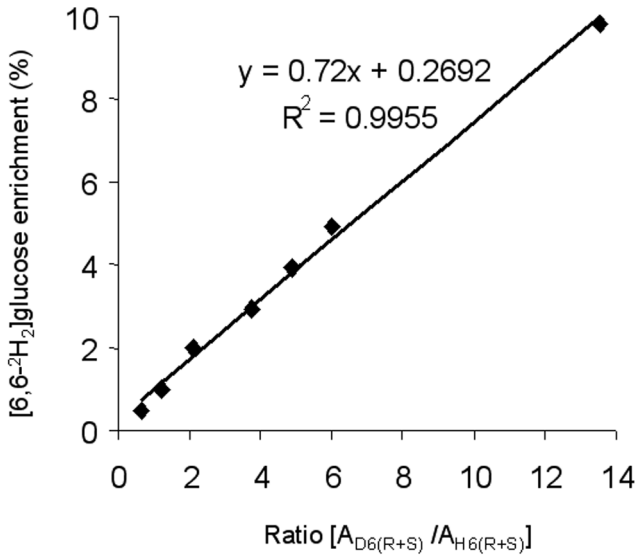

FIG. 2. [6,6-$^2H_2$]glucose enrichment versus ratio ($A_{D6(R+S)}/A_{H6(R+S)}$) obtained using a set of standard samples. Areas of $D6_{R+S}$ and $H6_{R+S}$ of glucose in the ratio are relative values after the reference peak (2.8 ppm) of DMF-d_7 was set to 100.

Table 1
Comparison of Tracer Enrichment, the Rate of GP, and Plasma Glucose Level from Two Groups (n = 6 for Each) of Rats That Received a Single Tracer, [6,6-$^2H_2$]Glucose or [3,4-$^{13}C_2$]Glucose

Tracer	Enrichment (%)	GP (μmol/kg/min)	Plasma glucose (mM)
[6,6-$^2H_2$]Glucose	1.87 ± 0.14	50.8 ± 3.9	7.1 ± 1.8
[3,4-$^{13}C_2$]Glucose	2.04 ± 0.04	45.4 ± 4.7	6.2 ± 0.8
P value	0.121	0.058	0.282

DISCUSSION

The values of GP determined here were all within the range of values, typically 32 to 67 μmol/kg/min, reported for fasted rats using various techniques (3,8,9–11). [6,6-$^2H_2$]glucose is currently one of the more widely accepted tracers for glucose turnover measurements. The validity of this tracer has been evaluated by Previs et al., who reported identical results by simultaneous infusion of [U-^{13}C]glucose and [6,6-$^2H_2$]glucose in rats with analysis by gas chromatography-mass spectrometry (1). It is worth noting, however, that not all tracers of glucose turnover yield the same results. For example, [2-^{2}H]glucose and [6,6-$^2H_2$]glucose are deuterium labeled tracers used in glucose turnover studies with detection by mass spectrometry. [2-^{2}H]Glucose is thought to overestimate glucose turnover because the deuterium can be lost by exchange between glucose-6-phosphate and fructose-6-phosphate which results in appearance of unlabeled plasma glucose. In fact, glucose turnover measured by [2-^{2}H]glucose (or the radioactive counterpart) has been compared to other measures of glucose turnover to specifically assess futile cycling between extracellular glucose and the glucose-6-phosphate pool (12,13). In contrast, the deuterium labels of [6,6-$^2H_2$]glucose are not lost during futile cycling, and for this reason [6,6-$^2H_2$]glucose is considered to report the true value of hepatic GP (14,15).

This study introduces a new method for the quantification of deuterium enrichment of plasma glucose. Sodium formate was proposed earlier as an internal reference for the quantification (7), but DMF-d_7 has significant advantages over formate. The methyl resonances of DMF-d_7 are reasonably sharp while the ^{1}H NMR signal of formate is rather broad, and this could contribute to errors in measurement of resonance areas. Of the two DMF methyl resonances (2.6 ppm and 2.8 ppm), the peak at 2.8 ppm was chosen in this work because a broad water peak often appears near 2.6 ppm in samples that require addition of more water than used here to increase the solubility of MAG (MAG has a limited solubility in neat acetonitrile). Another advantage is that DMF-d_7 can be easily removed from MAG by drying, which is an advantage if later processing of the sample is required.

Table 2
Comparison of Tracer Enrichment and the Rate of GP of Rats (n = 6) That Received both [6,6-$^2H_2$]Glucose and [3,4-$^{13}C_2$]Glucose Simultaneously

Tracer	Enrichment (%)	GP (μmol/kg/min)
[6,6-$^2H_2$]Glucose	2.20 ± 0.21	42.2 ± 4.2
[3,4-$^{13}C_2$]Glucose	2.23 ± 0.18	42.1 ± 3.7
P value	0.826	0.961

Note. The plasma glucose concentration for the group was 5.7 ± 0.6 mM.

[3,4-$^{13}C_2$]Glucose offers two critical advantages compared to other ^{13}C tracers of glucose. A basic assumption in measurement of glucose turnover is that a tracer molecule is not released from liver as the result of glycogenolysis or gluconeogenesis. A number of biochemical pathways could be followed by [3,4-$^{13}C_2$]glucose and it is important to consider whether [3,4-$^{13}C_2$]glucose (or other labeling patterns) could be generated. Oxidation of [3,4-$^{13}C_2$]glucose to pyruvate followed by decarboxylation to acetyl-CoA would yield only $^{13}CO_2$. Metabolism in the pentose phosphate pathway could yield complex labeling patterns in the triose pool, but only natural abundance singlets have been observed in positions 1, 2, 5, or 6, suggesting that this pathway does not impact the labeling patterns in plasma glucose. Metabolism of [3,4-$^{13}C_2$]glucose to the level of the triose pool followed by resynthesis could in principle regenerate [3,4-$^{13}C_2$]glucose (Fig. 3). However, [3,4-$^{13}C_2$]glucose represents at most about 2% of the glucose pool, so the chance that a molecule of [3,4-$^{13}C_2$]glucose was derived by degradation and resynthesis is ≤ 0.04%. [3,4-$^{13}C_2$]Glucose offers another, less obvious advantage: metabolism to pyruvate followed by carboxylation, further metabolism in the TCA cycle, and resynthesis to glucose cannot yield glucose labeled in any site other than positions 3 or 4 (Fig. 3). This is a significant advantage compared to alternative ^{13}C-tracers such as [1,6-$^{13}C_2$]glucose or [U-$^{13}C_6$]glucose, whose metabolism through pyruvate carboxylase could yield label throughout glucose produced by gluconeogenesis. This feature can be exploited because another ^{13}C-enriched gluconeogenic substrates that yield ^{13}C in positions 1, 2, 5, or 6 of glucose can be monitored simultaneously (4). The main disadvantage of [3,4-$^{13}C_2$]glucose is its high price because it is a novel tracer.

In summary, glucose turnover measured by NMR detection of [3,4-$^{13}C_2$]glucose was equivalent to results using a standard tracer of glucose turnover, [6,6-$^2H_2$]glucose. One significant advantage of [3,4-$^{13}C_2$]glucose is that carefully selected other ^{13}C tracers of TCA cycle metabolism can be used in the same experiment to probe multiple metabolic pathways (4). Furthermore, since NMR detection of either ^{13}C or ^{2}H is not sensitive to the presence of the other nucleus at the levels of enrichment typically used in these experiments, further metabolic information can also be obtained from a ^{2}H NMR spectrum of the same sample (4). Combining these tracers allows a relatively complete analysis of hepatic fluxes from a single blood sample, including GP, glycogenolysis, gluconeogenesis from glycerol, gluconeogenesis from phosphoenol pyruvate,

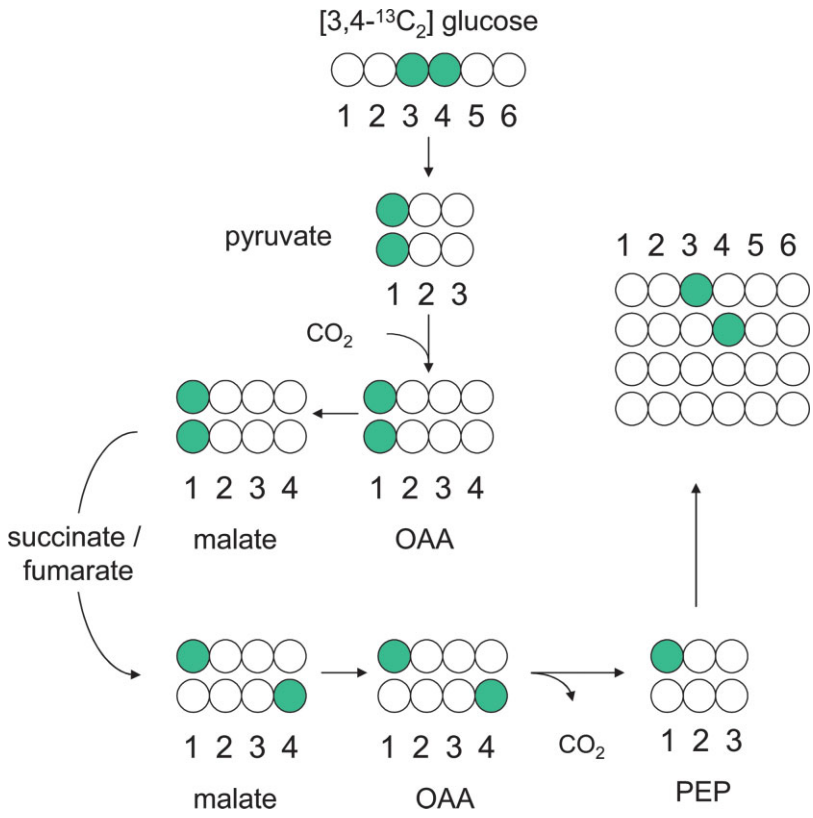

FIG. 3. Metabolism of [3,4-$^{13}C_2$]glucose through the citric acid cycle. If [3,4-$^{13}C_2$]glucose is metabolized to pyruvate followed by decarboxylation to acetyl-CoA, the ^{13}C label is lost as $^{13}CO_2$. Another possible fate, carboxylation to [1-^{13}C]oxaloacetate, is illustrated above. [1-^{13}C]Oxaloacetate is metabolized to [1-^{13}C]malate, which in turn is in equilibrium with fumarate and succinate. As the result of equilibration in the succinate and fumarate pool, a mixture of [1-^{13}C] oxaloacetate and [4-^{13}C]oxaloacetate is generated in a 1:1 ratio. Through decarboxylation, a mixture of [1-^{13}C]PEP and unenriched PEP is produced. After passing through gluconeogenesis in the presence of largely unenriched trioses, a mixture of [3-^{13}C]glucose, [4-^{13}C]glucose, and unenriched glucose is produced in a 1:1:2 ratio. The chance that [3,4-$^{13}C_2$]glucose could be resynthesized after metabolism of tracer [3,4-$^{13}C_2$]glucose in the TCA cycle is negligible because of dilution with unenriched TCA cycle intermediates.

and multiple fluxes in the TCA cycle (4). These methods are easily extended to patients with abnormal glucose metabolism.

ACKNOWLEDGMENTS

We thank Charles Storey and Angela Milde for their excellent work in animal experiments and Erin Smith for technical support.

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