An integrated $^2$H and $^{13}$C NMR study of gluconeogenesis and TCA cycle flux in humans

JOHN G. JONES, MICHAEL A. SOLOMON, SUZANNE M. COLE, A. DEAN SHERRY, and CRAIG R. MALLOY. An integrated $^2$H and $^{13}$C NMR study of gluconeogenesis and TCA cycle flux in humans. Am J Physiol Endocrinol Metab 281: E848–E856, 2001.—Hepatic glucose synthesis from glycogen, glycerol, and the tricarboxylic acid (TCA) cycle was measured in five overnight-fasted subjects by $^1$H, $^2$H, and $^{13}$C NMR analysis of blood glucose, urinary acetaminophen glucuronide, and urinary phenylacetylglutamine after administration of [1,6-$^{13}$C$_2$]glucose, $^2$H$_2$O, and [U-$^{13}$C$_3$]propionate. This combination of tracers allows three separate elements of hepatic glucose production (GP) to be probed simultaneously in a single study: 1) endogenous GP, 2) the contribution of glycogen, phosphoenolpyruvate (PEP), and glycerol to GP, and 3) flux through PEP carboxykinase, pyruvate recycling, and the TCA cycle. Isotope-dilution measurements of [1,6-$^{13}$C$_2$]glucose by $^1$H and $^{13}$C NMR indicated that GP in 16-h-fasted humans was 10.7 ± 0.9 μmol·kg$^{-1}$·min$^{-1}$. $^2$H NMR spectra of monoacetone glucose (derived from plasma glucose) provided the relative $^2$H enrichment at glucose H-2, H-5, and H-6S, which, in turn, reflects the contribution of glycogen, PEP, and glycerol to total GP (5.5 ± 0.7, 4.8 ± 1.0, and 0.4 ± 0.3 μmol·kg$^{-1}$·min$^{-1}$, respectively). Interestingly, $^{13}$C NMR isotopomer analysis of phenylacetylglutamine and acetaminophen glucuronide reported different values for PEP carboxykinase flux (68.8 ± 9.8 vs. 37.5 ± 7.9 μmol·kg$^{-1}$·min$^{-1}$), PEP recycling flux (59.1 ± 9.8 vs. 27.8 ± 6.8 μmol·kg$^{-1}$·min$^{-1}$), and TCA cycle flux (10.9 ± 1.4 vs. 5.4 ± 1.4 μmol·kg$^{-1}$·min$^{-1}$). These differences may reflect zonation of propionate metabolism in the liver.

monoacetone glucose; acetaminophen glucuronide; carbon 13; deuterium; gluconeogenesis; liver metabolism

THE LIVER PLAYS A PRINCIPAL ROLE in glucose homeostasis by regulating glucose synthesis and storage in response to the normal changes in daily nutritional and hormonal status. Under postabsorptive conditions, hepatic glycogenolysis and gluconeogenesis contribute to endogenous glucose production (GP) (6, 15–17, 23, 31, 32, 41, 44, 45). Because the majority of gluconeogenic carbons are derived from phosphoenolpyruvate (PEP) via the tricarboxylic acid (TCA) cycle (1, 6, 23, 31, 32), hepatic GP is also intimately linked to acetyl-CoA oxidation and energy production. Together, these biochemical pathways form a metabolic network (Fig. 1) that is highly responsive to matching the external demand for glucose with the availability of glycogen, gluconeogenic precursors, and energy. Measurements of carbon flux through this network typically combine an isotope dilution measurement of endogenous GP with additional tracer measurements of the contributing pathways (6, 8–10, 15, 18, 19, 25). As one example, GP in fasting humans was determined by analysis of the $^2$H enrichment at glucose C-6 using the hexamethylenetetramine method and mass spectrometry (MS) after infusion of [6,6-$^2$H$_2$]glucose at a known rate (6). It was possible to also give oral $^2$H$_2$O and measure the contribution of gluconeogenesis to GP from $^2$H enrichments at glucose C-5 vs. C-2 after parallel selective degradations of plasma glucose and analysis by MS (6). However, the contributions of glyceroL vs. TCA cycle intermediates to gluconeogenesis could not be differentiated in this experiment, nor can $^{13}$C tracers be combined with $^2$H tracers using the hexamethylenetetramine method.

$^{13}$C tracers have also been used to measure metabolic flux through the network. Endogenous GP was monitored with [U-$^{13}$C$_6$]glucose by following disappearance of the parent m+6 isotopomer from plasma glucose (23, 24, 38, 40, 41). Additional metabolic information can be derived from partially labeled glucose molecules generated by recycling of $^{13}$C label in this experiment. However, the extent to which the recycled label can be reliably analyzed and related to rates of gluconeogenesis and TCA cycle flux is controversial (24, 26, 28). Others have used tracers, such as [3-$^{13}$C]lactate, that enter at the level of the TCA cycle along with a separate tracer for measuring endogenous GP (9, 10). Neither [U-$^{13}$C$_6$]glucose nor [3-$^{13}$C]lactate tracers, however, can differentiate glucose produced from glycogen, glycerol, or the TCA cycle.

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In this report, we demonstrate that a $^2$H NMR spectrum of monoacetone glucose (42) may be used to measure the distribution of deuterium in blood glucose after ingestion of $^2$H$_2$O. This information allows the direct calculation of the contribution of glycogen, glycerol, and the TCA cycle to GP in humans. Although inherently less sensitive, the NMR method offers several advantages over MS analysis of glucose $^2$H enrichment (31, 32). First, it does not require carbon-by-carbon degradation of glucose; rather, the relative $^2$H enrichment at each carbon position of glucose can be read out in a single $^2$H NMR spectrum. Second, the prochiral H-6R and H-6S resonances are well separated in the $^2$H NMR spectrum of monoacetone glucose (42); thus the normal assumptions required by MS to quantitatively evaluate exchange at the level of fumarase in the TCA cycle are eliminated. This allows a separate measure of gluconeogenesis from the level of the triose phosphates (glycerol) vs PEP (the TCA cycle). Finally, the $^2$H measurement is not compromised by the presence of $^{13}$C tracers, so experiments can be designed to measure GP, gluconeogenic flux, pyruvate recycling flux, and TCA cycle flux in a single experiment. We illustrate the method here by reporting these flux values in 16-h-fasted humans using the combined tracers [1,6-$^{13}$C$_2$]glucose, $^2$H$_2$O, and [U-$^{13}$C$_3$]propionate. Thus a rather comprehensive picture of liver metabolism can be obtained during a single patient visit, making the technique highly suitable for routine clinical application.

**METHODS**

**Materials.** Cambridge Isotopes (Cambridge, MA) was our source for 99% $^2$H$_2$O, 99% [1,6-$^{13}$C$_2$]glucose, and 99% [U-$^{13}$C$_3$]propionate. Acetaminophen was derived from regular-strength Tylenol capsules, and phenylacetate was obtained from Sigma (St. Louis, MO).

**Experimental protocol.** Five healthy, nonobese subjects (2 men and 3 women, 21–36 yr of age, 50–86 (70 ± 14) kg body wt) were studied under a protocol approved by the institutional human studies committee. Subjects were admitted to the General Clinical Research Center at Parkland Hospital and examined (history and physical) by an internist. All had blood glucose levels in the normal range (70–110 mg/dl), and none reported a history of chronic illness or use of medications on a regular basis. All subjects began fasting at 6 PM. At 11 PM and again at 3 AM, 99% $^2$H$_2$O was taken orally (2.5 g/kg body water, calculated as total weight times 0.6 for men or 0.5 for women). During the remainder of the study, 0.5% $^2$H$_2$O was given ad libitum. At 6 AM, subjects ingested a tablet containing 325 mg of acetaminophen. Between 7 and 8 AM, subjects ingested tablets containing phenylacetate (20 mg/kg) and another 650 mg of acetaminophen. At 8 AM, a 3-h primed infusion of [1,6-$^{13}$C$_2$]glucose (167 mg, 1.67 mg/min) was initiated for each subject. The infusion medium was prepared by dissolving 500 mg of sterile and pyrogen-free [1,6-$^{13}$C$_2$]glucose into 150 ml of saline. The infusion medium was passed through a 0.22-μm filter during administration. Subjects received a 50-ml bolus of infusion medium over 2 min followed by constant infusion of 30 ml/h. Subjects also ingested [U-$^{13}$C$_3$]propionate (10 mg/kg, packaged into 3 gel caps) between 8 and 9 AM. Also, beginning at 8 AM, 10 ml of blood were drawn from a contralateral vein every 20 min for 2 h, with additional blood drawn at 2.5 and 3 h. This amounted to seven blood samples and a total of 70 ml of whole blood per subject. Urine was also collected every hour from 8 AM to 2 PM, at which point the study was concluded. The infusion medium was prepared by dissolving 500 mg of sterile and pyrogen-free [1,6-$^{13}$C$_2$]glucose into 150 ml of saline. The infusion medium was passed through a 0.22-μm filter during administration. Subjects received a 50-ml bolus of infusion medium over 2 min followed by constant infusion of 30 ml/h. Subjects also ingested [U-$^{13}$C$_3$]propionate (10 mg/kg, packaged into 3 gel caps) between 8 and 9 AM. Also, beginning at 8 AM, 10 ml of blood were drawn from a contralateral vein every 20 min for 2 h, with additional blood drawn at 2.5 and 3 h. This amounted to seven blood samples and a total of 70 ml of whole blood per subject. Urine was also collected every hour from 8 AM to 2 PM, at which point the study was concluded.

**Analytic procedures.** For each subject, an aliquot of the [1,6-$^{13}$C$_2$]glucose infusion medium was frozen and enzymatically assayed for glucose. Blood samples were chilled immediately after being drawn and centrifuged at 4°C in heparinized tubes. The plasma was then processed for $^{13}$C and $^1$H spectroscopy of plasma glucose by perchloric acid extraction, as previously described (21). For $^2$H NMR analysis of positional deuterium enrichment, two to three plasma extracts from each subject were pooled and lyophilized to complete dryness. Glucose was converted to monoacetone glucose by

![Fig. 1. Metabolic pathways involved in hepatic glucose synthesis and tricarboxylic acid (TCA) cycle activity. G-6-P, glucose 6-phosphate; G-3-P, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; FBP, fructose bisphosphate; PEP, phosphoenolpyruvate; OAA, oxaloacetate; Succ-CoA, succinyl-CoA; α-kG, α-ketoglutarate.](image-url)
use of the method of Landau et al. (31). After lyophilization, the residue containing monoacetone glucose was dissolved in 0.6 ml of 90% acetonitrile-10% 2H-depleted water plus a few grains of sodium bicarbonate (42), and insoluble material was centrifuged and discarded. Urine samples were treated with urease and β-glucuronidase and lyophilized as described previously (21). The extract was then reconstituted in 10 ml of water, and insoluble material was precipitated by centrifugation. The supernatant was adjusted to pH 1.0 with perchloric acid, and the sample was applied to an 8- to 10-ml cation-exchange column (Dowex-50 × 8-H +) followed by 40 ml of water. The column effluent was neutralized with KOH, lyophilized, and resuspended in 600 μl of 4H2O. The pH was then adjusted to 8.0 with NH4OH, the samples were centrifuged at 13,000 rpm with an Eppendorf centrifuge, and the supernatants were pipetted into 5-mm NMR tubes.

NMR spectroscopy. Proton-decoupled 13C NMR spectra of blood and urine extracts were collected using a Unity Inova 14.1-T spectrometer operating at 150.9 MHz. Free-induction decays were multiplied by a 0.1- to 0.2-Hz exponential function, defined by a Fourier transformation. Typically, 9,000–18,000 free-induction decays were summed for each blood extract and 6,000 for each urine extract, resulting in collection times of 5–14 h per extract. Proton-decoupled 2H NMR spectra of monoacetone glucose were acquired at 50°C (42) with the same probe using a 90° pulse and a sweep width of 920 Hz digitized into 992 points, giving an acquisition time of 0.512 s. No additional interpulse delays were used in this pulse sequence. Typically 90,000–100,000 scans were averaged. The data were zero-filled to 4 K, multiplied by a 1-Hz exponential function to increase signal-to-noise ratio, and Fourier transformed. 2H signal intensities obtained with these parameters were corrected for minor effects of differential saturation. A comparison of 2H signal areas in spectra collected using these standard pulsing conditions with those measured in spectra collected using a 1.0-s acquisition time [sufficient for complete relaxation (38)] were identical to within 7%. Nevertheless, small correction factors were used to allow for partial saturation when the 0.512-s acquisition conditions were used. 1H NMR spectra were obtained with the same spectrometer by means of a 5-mm indirect probe. Spectra were acquired with a 90° pulse after presaturation of the residual water signal and a 15-s interpulse delay. Long-range couplings between 13C C-2 to C-6 and H-1 were abolished by the application of a narrow-band WALTZ-16 13C-decoupling pulse sequence covering the 60- to 75-ppm region of the 13C NMR spectrum (18). Two hundred fifty-six acquisitions were collected for a total collection time of 64 min. All NMR spectra were analyzed using the curve-fitting routine supplied with the NUTS PC-based NMR spectral analysis program (Acorn NMR, Fremont, CA).

Metabolic flux calculations. The 13C enrichment in the [1,6,13C2]glucose used for the infusions was verified by 1H NMR. The fraction of blood glucose that contained 13C in C-1 was defined as $g$, the area of the doublet due to flux (JCH) in the glucose H-1 resonance relative to the total H-1 resonance area (Fig. 2). The fraction of blood glucose that was [1,6,13C2]glucose relative to all glucose containing 13C in C-1 was defined as $f$, the area of the doublet due to flux (JCH) arising from [1,5,13C2]glucose relative to the total area of the glucose C-1 resonance (Fig. 2). The fraction of [1,6,13C2] glucose in plasma glucose was calculated as $g$. The values measured in spectra collected at 120, 150, and 180 min were averaged for each subject and used in the calculation of GP. The rate of appearance of glucose was calculated from the known infusion rate of [1,6,13C2]glucose ($r$) divided by the average fraction found in plasma over the 120- to 180-min period. GP is then defined as the rate of appearance of glucose minus the rate of infusion of [1,6,13C2]glucose, or $GP = (r/fg) - r$.

The fraction of glucose derived from glycogen, PEP, and gluconeogenesis was estimated from the ratio of deuterium enrichment at positions 2, 5, and 6 as reported in the 2H NMR spectrum of monoacetone glucose (42) by use of the following equations:

- Glucose fraction from glycogen $= 1 - (H-5/H-2)$
- Glucose fraction from glycerol $= (H-5 - H-6S)/H-2$
- Glucose fraction from PEP $= H-6S/H-3$

Relative anaplerotic flux (OAA → PEP, where OAA is oxaloacetate), pyruvate recycling flux (PEP → pyruvate or equivalent pathway), and gluconeogenic flux (PEP → glucose) were calculated from the multiplet areas measured in the 13C NMR spectrum of urinary glucuronate or phenylacetylglutamine (PAGN), as described previously (18, 20, 21). For urinary glucuronate C-5 (the C-5β resonance was analyzed), the relevant equations are:

- OAA → PEP $= (C-5D56 - C-5D45)/C-5D45$
- PEP → pyruvate $= (C-5D56 - C-5Q)/C-5D45$
- PEP → glucose $= (C-5Q - C-5D45)/C-5D45$
- For PAGN C-2, the relevant equations are:

- OAA → PEP $= (C-2D23 - C-2D12)/C-2D12$
- PEP → pyruvate $= (C-2D23 - C-2Q)/C-2D12$
- PEP → glucose $= (C-2Q - C-2D12)/C-2D12$

Gluconeogenic flux from PEP is the difference between anaplerosis (OAA → PEP) and pyruvate recycling (PEP → pyruvate). PEP recycling is indicated here by PEP → pyruvate, although it should be noted that the combined pathway OAA → PEP → pyruvate cannot be distinguished from malate → pyruvate.

These relative fluxes were then converted to absolute values as follows. First, absolute fluxes in hexose units from
glycogen, PEP, and glycerol were defined as the product of each fractional contribution (Eqs. 1–3) times the endogenous GP in micromoles of glucose per kilogram per minute. Second, the rate of GP from PEP was converted to the rate of production of PEP by multiplying by 2. Finally, fluxes involved in the TCA cycle were calculated by indexing the relative fluxes (Eqs. 4–6 or 7–9) to the rate of production of PEP.

Statistical analysis. Values are means ± SD. Means were compared as noted using a t-test assuming unequal variances.

RESULTS

Each of the three separate components required for analysis of gluconeogenesis is presented individually.

Endogenous GP measurement from [1,6-13C2]glucose. A 13C NMR spectrum of the plasma glucose C-1 resonance from blood taken 180 min after administration of [U-13C3]propionate and [1,6-13C2]glucose is shown in Fig. 2. The resonance features well-resolved multiplets arising from 13C-13C splitting, reflecting the presence of multiply labeled glucose molecules. These include signals from glucose isotopomers generated from the gluconeogenic metabolism of [U-13C3]propionate (D12 and D123) in addition to the tracer amount of infused [1,6-13C2]glucose (D16). As previously demonstrated (18), the fraction of [1,6-13C2]glucose remaining in plasma at any time point can be quantified by measuring the contribution of [1,6-13C2]glucose to the C-1β resonance (13C spectrum) and the total 13C enrichment as reported in the H-1α resonance (1H spectrum). The 1H NMR spectrum of the H-1α proton features well-resolved 13C satellites with sufficient signal-to-noise ratio for reliable quantitation of the 2–3% excess 13C enrichment levels from this experiment. Figure 3 summarizes the [1,6-13C2]glucose fractional enrichment values obtained from serial blood sampling for the five subjects. The fractional enrichment of plasma [1,6-13C2]glucose reached steady state well before the end of the infusion, with enrichments of 0.75–1.35%. For each individual, the steady-state enrichment was calculated as the mean of the 120-, 150-, and 180-min enrichments. From these data, average endogenous GP for these five individuals was 10.7 ± 0.9 μmol·kg⁻¹·min⁻¹, with a range of 9.8–12.1 μmol·kg⁻¹·min⁻¹. These values are in good agreement with other measures of endogenous GP in healthy individuals after similar fasting times (the average of all values reported in Refs. 4, 6, 13–15, 23, 27, and 39 is 11.2 ± 1.7 μmol·kg⁻¹·min⁻¹).

Analysis of plasma glucose 2H enrichment by 2H NMR. Figure 4 shows a 2H NMR spectrum of monacetone glucose derived from two pooled plasma extracts (corresponding to 20 ml of whole blood). The area of each resonance is proportional to 2H enrichment at that position, so the spectrum provides a simple and direct readout of 2H enrichment ratios (42). It is important to point out that the 2H NMR measurement is reliable and quantitative for measurements of 2–3% excess 2H enrichment.

![Graph showing time course of plasma [1,6-13C2]glucose enrichment over the duration of [1,6-13C2]glucose infusion for 5 subjects.]

**Fig. 3.** Time course of plasma [1,6-13C2]glucose enrichment over the duration of [1,6-13C2]glucose infusion for 5 subjects.
not influenced by the presence of tracer levels of $^{13}$C in the glucose (or monoacetone glucose) molecule. Table 1 summarizes the relative contributions of glycogen, glycerol, and PEP to GP.

TCA cycle and gluconeogenic flux measurements from [U-$^{13}$C$_3$]propionate incorporation into hexose and PAGN. As previously demonstrated (21), relative anaplerotic, pyruvate recycling, and gluconeogenic fluxes can be obtained by a $^{13}$C isotopomer analysis of plasma glucose, urinary glucuronide, or the glutamine fragment in urinary PAGN. Equations 4–9 describe these relationships. Figure 5 illustrates typical multiplets observed in the $^{13}$C NMR spectrum of plasma glucose C-2$\beta$ and urinary glucuronate C-5$\beta$ of the same individual. The multiplet pattern arises from metabolism of [U-$^{13}$C$_3$]propionate at the level of the liver TCA cycle and is not affected by the presence or metabolism of [1,6-$^{13}$C$_2$]glucose (18). The difference in signal-to-noise ratio in these two spectra is largely due to the amount of urinary glucuronate in $\sim$100–150 ml of urine compared with the amount of glucose in 10 ml of blood. Given that the multiplets in blood glucose C-2$\beta$ and urinary glucuronate C-5$\beta$ report identical flux values (21) and given the large differences in signal-to-noise ratio of the spectra shown, relative flux values as reported by the glucuronate spectra are reported here. Estimates of flux ratios based on acetaminophen glucuronide for five healthy, nonobese individuals were reported as $\pm 21$. The $^{13}$C NMR spectra of urinary PAGN were also of high quality (Fig. 6). Analysis of the glutamine C-2 multiplets using Eqs. 7–9 provided the following relative flux estimates (Table 1): OAA $\rightarrow$ PEP = 6.3 $\pm$ 0.4, PEP $\rightarrow$ pyruvate = 5.4 $\pm$ 0.4, and PEP $\rightarrow$ glucose = 0.9 $\pm$ 0.2. As noted in an earlier study of 24- to 28-h-
fasted individuals (21), flux estimates determined by analysis of PAGN were significantly different from those derived from spectra of blood glucose or urinary glucuronate. A comparison of the flux ratios derived from acetaminophen glucuronide and PAGN (Table 1) shows a significantly lower relative PEP → glucose flux for PAGN than for glucuronate (P < 0.01). There was no significant difference in estimates of PEP → pyruvate or OAA → PEP (relative to citrate synthase, Table 1).

With the methylene carbon of phenylacetate (~44 ppm, 1.1% 13C) as an internal standard, the enrichment in PAGN C-2 and C-3 was 2.13 ± 0.19 and 2.04 ± 0.16%, respectively, while the 13C enrichment in C-4 was essentially equal to natural abundance levels (1.00 ± 0.06%). The enrichment in C-4 reports enrichment in acetyl-CoA and suggests that C-2 of acetyl-CoA was not enriched above natural abundance. However, the PAGN C-4 resonance did show a very small doublet (D45) characteristic of C-4 to C-5 coupling (52 Hz) resulting from entry of [1,2-13C2]acetyl-CoA into the TCA cycle. Because [1,2-13C2]acetyl-CoA can only arise from [1,2,3-13C3]- or [2,3-13C2]pyruvate, the presence of D45 indicates that a small portion of recycled pyruvate was oxidized to acetyl-CoA by pyruvate dehydrogenase. The intensity of the D45 signal was 5% of that of the C-2 multiplet, indicating that the level of acetyl-CoA labeling was very low compared with that of OAA, pyruvate, and PEP. However, because Eqs. 4–9 explicitly assume zero labeling of acetyl-CoA (20), this small enrichment of acetyl-CoA could potentially introduce errors into the flux estimates. The possible impact of this on anaplerosis, pyruvate recycling, and gluconeogenic flux estimates was tested by simulating (using tcaSIM1) 13C NMR spectra of glutamate for two metabolic situations, one with no labeling of acetyl-CoA and another with entry of 5% [1,2-13C2]acetyl-CoA. Identical flux values (within 5%) were obtained by analysis of the resulting glutamate C-2 multiplet areas with the use of Eqs. 7–9. We conclude that enrichment of glutamate C-4 at the level seen here in PAGN does not interfere with accurate measurement of the metabolic fluxes of interest.

Integration of metabolic data. The relative flux values reported by the [U-13C3]propionate and 2H2O tracers were converted to absolute flux values by referencing them to GP as reported by turnover of [1,6-13C2]glucose. This provides the rather comprehensive picture of TCA cycle-related fluxes and gluconeogenesis summarized in Table 2. Absolute flux estimates are reported for glucuronate- and PAGN-based 13C-isotopomer analyses.

**DISCUSSION**

This study demonstrates that a comprehensive metabolic profile of gluconeogenesis and TCA cycle activity may be obtained in humans with three stable isotope tracers and NMR analysis of blood and urine samples. Multiple tracers were required, because multiple experimental determinations are necessary to describe the known pathways involved in gluconeogenesis. These data were obtained after enrichment of total body water with 2H2O and two 13C tracers, [1,6-13C2]glucose to measure endogenous GP and [U-13C3]propionate as an index of TCA cycle activity.

1H NMR of monoacetone glucose. The 1H NMR spectrum of monoacetone glucose (42) derived from 20 ml of plasma glucose provided a convenient, direct measure of 2H enrichment at each site in the glucose molecule. Enrichment at H-2, reflecting isotopic equilibration of plasma glucose H-2 with body water (4, 8, 39), was highest in every sample, while enrichment at H-5 was ~50% of that at H-2. This indicates that glycogenolysis and gluconeogenesis contribute equally to endogenous GP after a 16- to 18-h fast. These observations agree with gas chromatography (GC)-MS measurements of H-5 and H-2 enrichments in 14- and 18.5-h-fasted individuals, where the contribution of gluconeogenesis to endogenous GP was reported to be 47 ± 4 and 54 ± 7.

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**Table 2. Fluxes through pathways supporting glucose production**

<table>
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<tr>
<th>Subject</th>
<th>Glucose Production, ( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} )</th>
<th>Rate of Production of Glucose from Each Source, ( \mu \text{mol} \cdot \text{hexose} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} )</th>
<th>Glucose</th>
<th>Glycerol</th>
<th>PEP</th>
<th>Fluxes from Analysis of Urine Acetaminophen Glucuronide, ( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} )</th>
<th>Fluxes from Analysis of Urine, PAGN, ( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} )</th>
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<td>4.52</td>
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Mean ± SD 10.7 ± 0.9 5.52 ± 0.67 0.35 ± 0.31 4.84 ± 1.03 37.45 ± 7.92 27.78 ± 6.77 5.38 ± 1.42 68.79 ± 9.79 59.11 ± 9.52 10.91 ± 1.41

Results from 2H NMR analysis of blood monoacetone glucose and the measured glucose production were used calculate the rate of glucose production from PEP and other sources. CS, citrate synthase. The rate of production of PEP from the TCA cycle (in triose units) was indexed to flux ratios (calculated from urine acetaminophen glucuronide or urine phenylacetylglutamine (PAGN) in the TCA cycle to calculate absolute fluxes. Numbers in parentheses refer to the numbered pathways of Fig. 1.
2\%", respectively (6, 31). Furthermore, the observation that $^2$H enrichment at H-6S was $\sim$95\% of that at H-5 indicates that the majority of gluconeogenic carbons are derived from the TCA cycle (PEP) and very few from glycerol (4, 8, 31). Most importantly, the $^2$H NMR spectrum of monoacetone glucose shows separate resonances for the prochiral H-6 hydrogens of glucose (42), while exchange of $^4$H$_2$O at the level of fumarase in the TCA cycle specifically enriches the H-6S position (46). Interestingly, the prochiral H-6 protons had comparable levels of enrichment, with a tendency toward higher enrichment in H-6S.

The capacity to measure individual enrichment of the prochiral glucose hydrogens by $^2$H NMR provides additional insight into the mechanisms that contribute to the enrichment of the methylene hydrogens of PEP from body water. Although the exchange mechanisms for enriching H-2 and H-5 of glucose from body water are considered to be essentially quantitative, exchange of pyruvate and water hydrogens is believed to be only 80\% complete (31). If this is true, then enrichment of the PEP methylene hydrogens would be less than that of body water, and the H-6-to-H-2 enrichment ratio of glucose as measured by GC-MS would underestimate the contribution of PEP to GP. However, this should only be true for glucose H-6R, because pyruvate enrichment is reported by the pro-S hydrogen of OAA\(^2\), while the pro-R hydrogen of this intermediate can undergo complete exchange with body water by interconversion with malate and fumarate (Fig. 7). If randomization between OAA, malate, and fumarate is complete, then $^2$H enrichment at the pro-R hydrogen of OAA would equal that of body water. Thus, to the extent that randomization is incomplete, enrichment of the pro-R hydrogen of OAA will be a weighted average of pyruvate and body water enrichments. However, given that carbon tracer data from this and other studies have demonstrated that there is extensive backward scrambling of OAA with fumarate as well as extensive recy- cling of PEP, pyruvate, and OAA in liver (9, 10, 19, 32), it is perhaps not surprising to find that glucose H-6R and H-6S had near-equivalent levels of $^2$H enrichment from $^2$H$_2$O. This observation means that the H-6-to-H-2 enrichment ratio reported by GC-MS (a single H-6 measurement is taken) and a $^2$H enrichment distributed equally between H-6R and H-6S is assumed) should be equivalent to the H-6S-to-H-2 ratio reported by $^2$H NMR.

**TCA cycle fluxes.** In this study, PEP carboxykinase and pyruvate recycling were about seven- and fivefold higher than TCA cycle flux, while net gluconeo- genic outflow (defined as PEP $\rightarrow$ glucose flux) was about two-and-a-half times TCA cycle flux. Compared with 24- to 28-h-fasted individuals (19), gluconeogenic outflow in the present group (fasted for 16–18 h) tended to be lower, while PEP carboxykinase and pyruvate recycling fluxes tended to be higher.

\[
\begin{align*}
\text{Pyruvate} & \rightarrow \text{Oxaloacetate} \\
\text{PEP} & \rightarrow \text{Malate} \\
\text{Fumarate} & \rightarrow \text{Citrate}
\end{align*}
\]

\[
\begin{align*}
(1) & \quad \text{H} & \quad \text{C} & \quad \text{CO}_2 & \quad \text{O}_2 & \quad \text{C} & \quad \text{CO}_2 \\
(2) & \quad \text{H} & \quad \text{C} & \quad \text{CO}_2 & \quad \text{O}_2 & \quad \text{C} & \quad \text{CO}_2 \\
\end{align*}
\]

Fig. 7. Labeling of the prochiral methylene hydrogens of PEP from pyruvate and body water in the absence (1) and presence (2) of exchange between OAA, malate, and fumarate.

$^*$Labeled hydrogen derived from labeled water as a result of fumarase activity. $^\circ$, labeled hydrogen derived from pyruvate.

Estimates of relative PEP $\rightarrow$ glucose flux show a large variation between different studies. In the study of Magnusson et al. (35), PEP $\rightarrow$ glucose flux was approximately three times citrate synthase flux for 60-h-fasted individuals and overnight-fasted subjects given intravenous glucose and glucagon. In compari- son, the values reported by Diraison et al. (9, 10) are only $\sim$0.5 times flux through citrate synthase. Absolute TCA cycle fluxes are derived by indexing relative fluxes to an independent measurement of absolute PEP $\rightarrow$ glucose flux; therefore, uncertainties in relative PEP $\rightarrow$ glucose flux propagate systematic uncertain- ties in estimates of absolute TCA cycle fluxes. For example, absolute hepatic citrate synthase fluxes estimated by Diraison et al. range from 20 to 35 \(\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}\), while estimates\(^3\) from Magnusson et al. range from 5 to 7 \(\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}\). Absolute citrate synthase flux estimates from our study (5.4 and 10.9 \(\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}\) from urinary glucuronate and PAGN, respectively) are similar to the estimates of Magnusson et al. No other published estimates of human hepatic citrate synthase flux are available for comparison.

The anaplerotic, PEP recycling, and TCA cycle fluxes reported here are based on a simple set of equations that are valid when excess enrichment of acetyl-CoA and C-4 and C-5 of PAGN are negligible relative to enrichment of C-1 to C-3 (18). The advantage of such equations over computational analysis is that they provide a simple and direct way of relating the C-2 multiplet pattern from the $^{13}$C NMR spectrum of glucose or PAGN to metabolic fluxes through anaplerotic.

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\(^2\)Published flux estimates in millimoles per minute were converted to micromoles per kilogram per minute using the reported average weight of the subjects.

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whole blood was required for the 2H NMR measure-
ments of hepatic arteriovenous substrate balances
quire correlation of metabolic flux estimates with mea-
surements of plasma glucose under conditions where total
body water is enriched to 0.5% in 2H. Although this
amount of blood can be safely collected from adults, this
volume would be excessive for small children. Thus, for an equivalent study in children, one would be
required to increase the NMR sensitivity by using
smaller-volume 2H microprobes, or perhaps cryo-
probes, or by collecting larger amounts of glucose
equivalents via urinary glucuronide (31). Although
~1.0 mmol of glucuronide might be available in urine
(compared with ~0.11 mmol from 20 ml of blood), this
must be weighed against the loss of 2H data from the
H-6 position (glucose C-6 becomes a carboxylate in the
glucuronide) and the additional synthetic steps in-
volved in the conversion of acetaminophen glucuronide
to monoacetone glucose. Clearly, the preference would
be to perform the 1H analysis on plasma glucose when-
ever possible.

In summary, any comprehensive analysis of gluco-
neogenesis in humans requires multiple tracers. Exis-
ting protocols typically require experimental studies at
different times to account for the complex pathways
involving the TCA cycle. The combined 2H-13C NMR
isotopomer method reported here allows a practical
clinical method for measuring gluconeogenesis in a
single study. Furthermore, all the experimental data
are collected from blood or urine, and placing humans
into magnets is not required. Given that the metabolic
tracers can be administered orally and that all data
can potentially be collected from urinary metabolites,
this method now offers, for the first time, the possi-
Bility of a noninvasive examination for outpatient studies
that may prove valuable in population-based studies of
glucose metabolism.

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