Measurement of Fractional Whole-Body Gluconeogenesis in Humans From Blood Samples Using 2H Nuclear Magnetic Resonance Spectroscopy


Several problems limit quantification of gluconeogenesis. We applied in vitro 2H-nuclear magnetic resonance (NMR) spectroscopy to simultaneously measure 2H in all glucose carbons for direct assessment of gluconeogenesis. This method was compared with 2H measurement in carbons 5 and 2 using gas chromatography–mass spectrometry (hexamethylenetetramine [HMT]) and with in vivo 13C magnetic resonance spectroscopy (MRS). After 14 h of fasting, and following 2H2O ingestion, blood was obtained from nine healthy and seven type 2 diabetic subjects. Glucose was purified, acetylated, and analyzed for 2H in carbons 1–6 with 2H-NMR. Using 5:2 ratios, gluconeogenesis increased (P < 0.05) over time and mean gluconeogenesis was lower in control subjects than in type 2 diabetic patients (63 ± 3 vs. 75 ± 2%, P < 0.01). 13C-MRS revealed higher hepatic glycogenolysis in control subjects (3.9 ± 0.4 vs. 2.3 ± 0.2 μmol · kg−1 · min−1) yielding mean contribution of gluconeogenesis of 65 ± 3 and 77 ± 2% (P < 0.005). Measurement of gluconeogenesis by 2H-NMR correlated linearly with 13C-MRS (r = 0.758, P = 0.0007) and HMT (r = 0.759, P = 0.0007). In an additional protocol, 2H enrichments demonstrated a fast decline of gluconeogenesis from ~100 to ~68% (P < 0.02) within 4 h of galactose infusion after 40–44 h of fasting. Thus, in vitro 2H-NMR offers an alternative approach to determine fractional gluconeogenesis in good agreement with standard methods and allows monitoring of rapid metabolic alterations. Diabetes 52:2475–2482, 2003

In type 2 diabetic patients, gluconeogenesis primarily accounts for increased endogenous glucose production (EGP) and fasting plasma glucose concentrations (1). Previous methods for measuring gluconeogenesis yielded a wide range of estimates in humans, which may relate to biochemical limitations of those methods (2–5). Gluconeogenesis can be also calculated from the difference between the rates of EGP determined with [6-2H]glucose and of liver glycogen breakdown determined by in vivo 13C-nuclear magnetic resonance spectroscopy (MRS) (1,6). This method quantifies rates of gluconeogenesis but requires patients to lie for hours within a magnet (7). An alternative method is based on incorporation of 2H into glucose after 2H2O administration (8). In humans, gluconeogenesis can be calculated from 2H enrichments in hexamethylenetetramine (HMT) derived from blood glucose measured with gas chromatography–mass spectrometry (9,10). Briefly, 2H is incorporated into position 3 of phosphoenolpyruvate (PEP) during the equilibration of pyruvate with alanine and via conversion of the pyruvate to oxaloacetate to the extent it equilibrates with fumarate. The carbon at position 3 of PEP becomes carbon 6 of glucose. 2H is incorporated into position 2 of glyceraldehyde-3-P via its isomerization with dihydroxyacetone-3-P and via hydration of PEP to 2-phosphoglycerate. During glycerol conversion, 2H is introduced at position 2 of glyceraldehyde-3-P. Carbon two of glyceraldehyde-3-P becomes carbon 5 of glucose. Subsequently, position 2 of glucose is labeled with 2H during both gluconeogenesis and glycogenolysis. Thus, the ratio of 2H enrichment at position 5 to that at two of glucose represents the fractional contribution of gluconeogenesis to EGP (9,10). This method was shown to overcome limitations of previous methods in humans but requires extensive and time-consuming biochemical preparations.

Determination of 5:2 ratios by in vitro 2H-NMR spectroscopy using monoacetone glucose (11–14) has been reported. Biochemical short-comings of previous approaches and invasive or time-consuming procedures can be avoided by this method. In the present study, 5:2 ratios were obtained by an alternative approach applying in vitro 2H-NMR on a glucopyranosyl bromide derivative. The estimates of gluconeogenesis were compared with both in vivo 13C-MRS and the HMT method. This method was tested under both the usually applied
conditions of continuously increasing gluconeogenesis (i.e., overnight and prolonged fasting) and the conditions of rapidly decreasing fractional contribution of gluconeogenesis to EGP (i.e., intravenous galactose infusion after prolonged fasting). In the prolongedfasted state, gluconeogenesis almost completely accounts for EGP, butsince galactose in liver is converted to glucose via glucose-1-P and glucose-6-P (the same intermediates as in glycolysis), galactose administration is expected to cause a rapid fall in the contribution of gluconeogenesis to EGP.

**RESEARCH DESIGN AND METHODS**

**Clinical protocols**

**Study on the evaluation of the method.** Nine healthy male volunteers (control subjects) (age range 22–28 years, BMI 23.7 ± 0.5 kg/m², and HbA₁c 5.0 ± 0.1%) and seven well-controlled type 2 diabetic patients (age range 49–62 years, BMI 26.1 ± 0.9 kg/m², and HbA₁c 7.1 ± 0.2%) were on an isocaloric diet (>50% carbohydrates) and refrained from physical exercise for ≤3 days. The patients discontinued oral hypoglycemic agents at least 3 days before the study. None of them suffered from diabetes-related complications. All participants gave informed written consent to the protocols, which were approved by the institutional ethics board, and all participants participated in two protocols in randomized order. They ingested a liquid meal (50% carbohydrate, 20% fat) at 6:00 a.m. and fasted overnight. On day 1, they drank 5 g H₂O (99.9% H; Cambridge Isotope Laboratory, Andover, MA) per kilograms of body weight divided into four doses spaced 30 min from 6:00 (12 h of fasting) to 7:30 a.m. (10:16). Blood was drawn at 6:00, 8:00, 14 h (of fasting), and 10:00 (16 h of fasting) a.m. and at the end of the study at noon (18 h of fasting). The volume of blood samples was 30 ml per time point and was doubled in some experiments for determination of the precision of the method. No side effects of H₂O were observed.

On another day, subjects first received an intravenous bolus, adjusted for plasma glucose concentration, and from 6:00 until noon (18 h of fasting) they received a continuous infusion of [6,6-²H₂]glucose (99% enriched; H; Omnicron Biochemicals, South Bend, IN) at 0.5 mmol/l. After 18 h, [2-²H]glucose (98% enriched in H; Cambridge Isotope Laboratory, and perdeuterated glucose (98% enriched in H; Sigma Chemical, Perth, Australia). Signal intensities were not different for acquisition times between 1.5 and 3 s, which were used in all further experiments. To reduce line-broadening in unlocked mode occurring during acquisition of spectra for 5–20 h, the transmitter frequency was adjusted by the shift of the solvent resonance after each set of 2,048 transients. This yielded a resolution of 1.5 Hz, even after 36 h. All spectra were multiplied with an exponential window function of 1.5-Hz line broadening before Fourier transformation. Spectra were least-square fitted with Lorentzian lines using the Varian NMR deconvolution routine (17). The natural line widths of the glucose resonances were 7–8 Hz, whereas that of the solvent, benzene, was 3.6 Hz. Spectra were acquired at a temperature of 303 K. The percent contribution of gluconeogenesis to EGP was set to 100 times the ratio of H enrichment at position 5 to that at carbon 2 (5/2) of blood glucose (10).

In vivo ¹³C MRS. Subjects were lying within a magnet (3-T Medspec 3000-DBX system; Bruker Medical, Ettlingen, Germany) using a 10-cm double-tuned ¹H/¹³C circular coil (15,18). Spectra were acquired using a modified 1n inversion-based sequence (6) without H decoupling (19). Glyco-
gen concentrations were measured by integration of the carbon 1 glycerol doublet at 100.5 ppm using the same frequency bandwidth (±300 Hz) for all spectra. Absolute quantification was performed by comparing the peak integral with that of a glycerol standard (6). Corrections for loading and sensitive volume of the coil were done. Liver volumes were measured in a 1.5-T Vision imager (Siemens, Germany) using a body array coil and in-phase and postphase multislice FLASH imaging sequences (15,18).

Gas chromatography-mass spectrometry. H enrichments at positions 2 and 5 of blood glucose were determined by isolating the hydrogens in formic acid derived from HMT (9,10,20). Briefly, deproteinized blood samples were deionized by ion exchange, and the glucose was purified by high-performance liquid chromatography. Aliquots of glucose were converted to arabinol-5-P and ribitol-5-P and to xylose, which were oxidized with periodate to yield formic acid containing carbons 2 and 5 of glucose, respectively. The HMTs were assayed for H enrichments by mass spectrometry using mass 141 for the enrichments at positions 2 and 5.

To assess EGP, H enrichments in carbon 6 of glucose pentacacetate were assayed on a Hewlett-Packard 5,890 gas chromatograph interfaced to a Hewlett-Packard 5971A mass selective detector (15,18). M + 2 enrichments were measured from the mass-to-charge ratio of 202/200 of the fragment ion consisting of carbons 2 and 5 of glucose, respectively. The HMTs were assayed for H enrichments by mass spectrometry using mass 141 for the enrichments at positions 2 and 5.

H enrichments in plasma water were measured by Metabolic Solutions (Nashua, NH) using an isotopic ratio mass spectrometer.

**Metabolites and hormones.** Plasma glucose was measured on a Glucose Analyzer II (Beckman, Fullerton, CA). Plasma free fatty acids (FFAs) (Wako Chemicals, Neuss, Germany) were determined by in-house and inter-assay coefficient of variation (CV) <6%, lactate (CV 3.3%), and β-hydroxybutyrate (CV <0%) were quantified enzymatically. Plasma insulin, C-peptide, glucagon, and cortisol were measured by radioimmunoassay as described (15,16,22).

**Data analysis.** Data are presented as means ± SE unless otherwise stated. Linear regression analysis was performed by least-square fitting of data. One-way ANOVA with Bartlett’s test for equal variances and paired Student’s t tests were used as appropriate.

**RESULTS**

**Study on the evaluation of the method**

**Metabolites and hormones.** Plasma glucose was higher in type 2 diabetic patients (9.9 ± 1.1 mmol/l) than in control subjects (5.4 ± 0.1 mmol/l; P < 0.005). Type 2 diabetic patients also exhibited increased plasma concentrations of FFA (0.48 ± 0.03 vs. 0.35 ± 0.03 mmol/l; P < 0.001) and lactate (1.26 ± 0.12 vs. 0.50 ± 0.09 mmol/l; P < 0.01) but not β-hydroxybutyrate (0.12 ± 0.01 vs. 0.11 ± 0.01 mmol/l). Mean plasma insulin (60 ± 12 vs. 39 ± 13 pmol/l) and C-peptide (749 ± 149 vs. 484 ± 44 pmol/l) were higher (P < 0.005) in type 2 diabetic patients than in control subjects. Mean plasma glucagon (81 ± 10 vs. 70 ± 5 pmol/l), cortisol (337 ± 36 vs. 375 ± 37 nmol/l), and...
growth hormone (1.8 ± 0.7 vs. 1.0 ± 0.4 μg/l) were comparable between type 2 diabetic patients and control subjects. All of these parameters were not different between both days.

**Precision of in vitro $^2$H-NMR.** After 10 h of accumulation of spectra in a sample containing 91 mg 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide, the signal-to-noise (s/n) ratio of the smallest peak, i.e., resonance at carbon 6’, was ~20 for acquisition times of 3 s (12,000 transients).

To assess the reproducibility, spectra were recorded under identical conditions yielding an SD <3% for 5:2 ratios. At lowest s/n ratio of ~10 (for the resonance at carbon 6’), the SD of the 5:2 ratio increased to ≤6%.

To estimate the accuracy of the NMR measurements as a function of the s/n ratio, 2 mg 2,4,6-tetra-O-acetyl-α-D-[2-$^2$H]glucopyranosyl bromide were dissolved in natural abundance benzene, and four sets of $^2$H spectra were recorded with increasing numbers of transients (4, 16, 32, 64, 128, 256), giving a total of 24 measurements. The s/n ratio of the glucose signal ranged between 8 and 64 (4 and

---

**FIG. 1.** $^2$H-NMR spectra of 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide obtained in a single blood sample of one healthy volunteer at 14, 16, and 18 h of fasting upon ingestion of $^2$H$_2$O (5 g/kg body water).
**TABLE 1**

<table>
<thead>
<tr>
<th>1/2</th>
<th>3/2</th>
<th>4/2</th>
<th>5/2</th>
<th>6/2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control subjects</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 h</td>
<td>73 ± 4</td>
<td>49 ± 8</td>
<td>75 ± 4</td>
<td>51 ± 5</td>
</tr>
<tr>
<td>16 h</td>
<td>67 ± 5</td>
<td>42 ± 6</td>
<td>73 ± 7</td>
<td>65 ± 4</td>
</tr>
<tr>
<td>18 h</td>
<td>81 ± 3</td>
<td>53 ± 6</td>
<td>84 ± 6</td>
<td>73 ± 5</td>
</tr>
<tr>
<td>14–18 h</td>
<td>74 ± 3</td>
<td>49 ± 6</td>
<td>81 ± 4</td>
<td>63 ± 3</td>
</tr>
<tr>
<td><strong>Type 2 diabetic subjects</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 h</td>
<td>81 ± 3</td>
<td>43 ± 6</td>
<td>73 ± 3</td>
<td>62 ± 2</td>
</tr>
<tr>
<td>16 h</td>
<td>81 ± 5</td>
<td>42 ± 4</td>
<td>85 ± 6</td>
<td>75 ± 3</td>
</tr>
<tr>
<td>18 h</td>
<td>84 ± 2</td>
<td>42 ± 2</td>
<td>88 ± 3</td>
<td>87 ± 2</td>
</tr>
<tr>
<td>14–18 h</td>
<td>82 ± 3</td>
<td>45 ± 2</td>
<td>82 ± 3</td>
<td>75 ± 2</td>
</tr>
</tbody>
</table>

Data are means ± SEM.

The precision of data processing was determined by integrating the $^2$H peaks three times in two different samples, yielding a variance <2%.

Absolute enrichments were checked with solutions containing $^2$H-glucose and $^2$H-glucopyranosyl bromide exhibiting well-resolved $^2$H signal at positions 1, 2, 3, 4, and 6, whereas those at 5 and 6 were superimposed (Fig. 1). $^2$H spectra of 3-O-acetyl-1,2-5,6-di-O-isopropylidene-$\alpha$-$\beta$ glucosuranosyl bromide allowed separation of signals at positions 6 and 6', whereas those at 2 and 5 overlapped. $^2$H enrichments at positions 6 and 6' were identical (ratio at 6:6' 1.03 ± 0.08). Thus, for the glucopyranosyl bromide derivative, the integral of the resonance at carbon 5 was obtained by subtracting the $^2$H enrichments at position 6' from the combined resonances at carbons 5 and 6. This allowed us to quantify $^2$H enrichments at each of the seven positions and to calculate $^2$H incorporation into glucose. Because the glucopyranosyl bromide derivative is obtained in a yield of 75–95% in a simple two-step reaction, we used it as the compound of choice.

From the 5:2 ratios of $^2$H enrichments, gluconeogenesis increased from ∼62 and ∼51% at 14 h to ∼87 ($P < 0.001$) and ∼73% ($P = 0.005$) at 18 h of fasting in type 2 diabetic patients and control subjects, respectively (Table 1, Fig. 2). Type 2 diabetic patients presented with higher gluconeogenesis than control subjects ($P < 0.01$). Mean gluconeogenesis over the whole study period (14–18 h fasting) was ∼75% in type 2 diabetic patients and ∼63% in control subjects. In both type 2 diabetic patients and control subjects, the 3:2 and 6:2 ratios were lower ($P < 0.001$), whereas the 1:2 and 4:2 ratios were higher ($P < 0.001$) than the 5:2 ratios (Table 1). Using the HMT method, the 5:2 ratios gave an estimate of gluconeogenesis of 60 ± 3 and 50 ± 3% at 16 h and 67 ± 3% as well as 51 ± 3% at 18 h in type 2 diabetic patients and control subjects ($P < 0.001$), respectively (mean 63 ± 3 and 51 ± 3%, $P < 0.005$ vs. NMR method) (Table 2).

**Gluconeogenesis from in vivo 13C MRS.** Hepatic glycogen concentrations were lower ($P < 0.0001$) in type 2 diabetic patients at 12.5–13.5 h (210 ± 8 vs. 244 ± 6 mmol/l liver) (Fig. 2). In all individuals of both groups, glycogen declined linearly (type 2 diabetic patients $r^2 = 0.73 ± 0.07$, $P < 0.01$; control subjects $r^2 = 0.85 ± 0.04$, $P < 0.01$) and was also lower in type 2 diabetic patients at 17–18 h of fasting (175 ± 7 vs. 197 ± 6 mmol/l liver, $P < 0.0001$). Mean rates of glycogen breakdown were also lower in type 2 diabetic patients than in control subjects (2.3 ± 0.2 vs. 3.9 ± 0.4 μmol·kg body wt $^{-1}$·min $^{-1}$, $P < 0.005$) (Table 2). Rates of EGP were higher in type 2 diabetic patients (16.3 ± 1.2 vs. 11.2 ± 0.4 μmol·kg $^{-1}$·min $^{-1}$, $P < 0.001$) (Table 2). The difference between EGP and glycogenolysis gives the absolute rate of gluconeogenesis (7.9 ± 0.6 and 7.2 ± 0.5 μmol·kg body wt $^{-1}$·min $^{-1}$, respectively) which accounted for 85 ± 2 and 65 ± 3% of EGP, respectively ($P < 0.0005$ type 2 diabetic patients vs. control subjects) (Table 2). Individual data of body weight (type 2 diabetic patients 85 ± 5 kg and control subjects 73 ± 2 kg) and liver volume (1.62 ± 0.04 and 1.59 ± 0.04 l, respectively) were used to calculate rates of gluconeogenesis and glycogenolysis.

Estimates of the contributions of gluconeogenesis to EGP from in vitro $^2$H-NMR correlated linearly with that from in vivo $^{13}$C-MRS ($r = 0.757$, $P = 0.0007$) (Fig. 3). In vitro $^2$H-NMR also linearly correlated with HMT measurements ($r = 0.759$, $P = 0.0007$). Finally, in vivo $^{13}$C-MRS and HMT measurements were linearly related ($r = 0.713$, $P = 0.002$).

**Study on the effect of galactose Metabolites and hormones.** Plasma glucose declined ($P < 0.05$) from 5.1 ± 0.1 mmol/l at 12 h to 4.1 ± 0.1 mmol/l at 40 h. Thereafter, plasma glucose rose by ∼24% ($P < 0.01$) during galactose infusion (Fig. 4). Plasma insulin concentrations were 17 ± 1 pmol/l at 40 h and 27 ± 5 pmol/l at 44 h. During galactose infusion, plasma C-peptide increased from 245 ± 35 to 428 ± 59 pmol/l ($P < 0.05$). Plasma FFAs were 0.70 ± 0.12 mmol/l at 12 h, 0.86 ± 0.06 mmol/l at 40 h, and 0.73 ± 0.08 mmol/l at 44 h.

**Glycogen metabolism from in vivo $^{13}$C MRS.** Liver glycogen concentrations were 86 ± 4 mmol/l liver at the
37- to 38-h fast and declined \((P < 0.005)\) to \(77 \pm 3\ \text{mmol/l}\) at \(39–40\ h\) (Fig. 4), giving rates of hepatic glycogenolysis of \(59 \pm 12\ \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}\) at \(40\ h\) so that gluconeogenesis measured by in vivo \(^{13}\text{C}-\text{MRS}\) was \(89 \pm 2\%\) at \(37–40\ h\) of fasting. During galactose infusion, liver glycogen concentrations rose from \(78 \pm 9\ \text{mmol/l}\) \((40–41\ h)\) to \(104 \pm 11\ \text{mmol/l}\) \((41.5–42.5\ h,\ P < 0.0005)\) and \(109 \pm 10\ \text{mmol/l}\) \((43–44\ h,\ P < 0.0001)\), giving rates of hepatic glycogen synthesis of \(3.1 \pm 0.6\ \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}\). EGP remained constant during galactose infusion (mean 8.8 \pm 0.2 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}).

**Gluconeogenesis from \(^2\text{H}\) enrichments.** Using the HMT method, gluconeogenesis contributed to EGP by 94\% at 40 h \((P < 0.005)\) and declined to 72\% (42 h, \(P < 0.05\)) and 69\% (44 h, \(P < 0.005\)) during galactose infusion. Using in vitro \(^2\text{H}-\text{NMR}\), gluconeogenesis contributed to EGP by 99\% at 40 h and declined to

### TABLE 2

Summary of EGP, hepatic glycogenolysis, and gluconeogenesis in 7 type 2 diabetic and 9 nondiabetic volunteers

<table>
<thead>
<tr>
<th>Type 2 diabetic subjects</th>
<th>Control subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute rate ((\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}))</td>
<td>Absolute rate ((\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}))</td>
</tr>
<tr>
<td>Fraction of EGP (%)</td>
<td>Fraction of EGP (%)</td>
</tr>
<tr>
<td>------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>EGP</td>
<td>16.3 \pm 1.2*</td>
</tr>
<tr>
<td>Glycogenolysis</td>
<td>2.3 \pm 0.2*</td>
</tr>
<tr>
<td>GNG ((^{13}\text{C}-\text{MRS}))</td>
<td>14 \pm 1.3*</td>
</tr>
<tr>
<td>GNG ((^{2}\text{H}-\text{NMR}))</td>
<td>—</td>
</tr>
<tr>
<td>GNG (HMT)</td>
<td>—</td>
</tr>
<tr>
<td>Glucose</td>
<td>100</td>
</tr>
<tr>
<td>Gluconeogenesis</td>
<td>15 \pm 2*</td>
</tr>
<tr>
<td>GNG (HMT)</td>
<td>75 \pm 2*</td>
</tr>
</tbody>
</table>

Data are means \pm SEM. Comparison of estimates of gluconeogenesis (GNG) as calculated from 5:2 ratios in plasma glucose measured with in vitro \(^2\text{H}-\text{NMR}\) spectroscopy, from HMT and from the difference between EGP and hepatic glycogenolysis measured with in vivo \(^{13}\text{C}-\text{MRS}\).

\(*P < 0.01 \text{ vs. control subjects}\) \(\dagger P < 0.005 \text{ vs. HMT}\).
79 ± 2 and 70 ± 3% (44 h, \( P < 0.02 \)) during galactose infusion (Fig. 4). Estimates for gluconeogenesis were not different between both methods.

**DISCUSSION**

In vitro \(^{2}H\)-NMR has recently been used to measure \(^{2}H\) incorporation from \(^{2}H\)O in glucose from various natural sources. In one study (23), glucose, isolated from plants, was converted to 3,6-anhydro-1,2-O-isopropylidene-\(\alpha\)-D-glucopyranose. That derivative exhibited all seven separated \(^{2}H\) resonances at 61 MHz, but multiple preparation steps prevent its efficient application on a micro scale. In another study (12), glucose, isolated from rat plasma, was oxidized to its gluconate. \(^{2}H\) at carbon 1 is lost during the conversion, and resolved signals are only obtained for carbons 2 and 3. The 3:2 ratio was then used to estimate gluconeogenesis. The same group reported fully resolved \(^{2}H\) resonances employing monoacetone glucose (11) using body water enrichments of \(^{2}H\) between 2.5–5% in rats. More recently, this method was used to estimate gluconeogenesis in humans at body water enrichments of 0.5% (13). We found our derivative to be easier to prepare and provide a higher overall yield. However, the resolution of monoacetone glucose allows the baseline separation of all \(^{2}H\) resonances at 10 T, and the line width of NMR signals was slightly superior in our hands.

The present study also used \(^{2}H\) enrichments of 0.5% in body water as reported previously in other studies in humans (9,10,13,16). These procedures make determination of \(^{2}H\) enrichments at all carbons of glucose feasible at a safe dose of \(^{2}H\)O in humans. Although the \(^{1}H\) resonances at carbons 5 and 6 of glucose overlapped, identical \(^{2}H\) enrichments at positions 6 and 6’—which is in agreement with studies in rats (11)—allowed us to calculate 5:2 ratios. Our estimates of gluconeogenesis are similar to those reported at 15 and 18.5 h of fasting (24) but somewhat higher than in other studies (22,23) using the HMT method. Measurements of gluconeogenesis by the HMT method and in vitro \(^{2}H\)-NMR were linearly correlated, although the latter method gave higher estimates of 5:2 ratios. As both methods measure 5:2 ratios in blood glucose, no differences are to be expected. One potential explanation for the higher estimates from in vitro \(^{2}H\)-NMR could reside in the calculation of 5:2 ratios, which requires subtracting the peak area at position 6’ from that at 5 and 6 in order to obtain the peak area at position 5. Although the peak areas at positions 6 and 6’ of glucopyranosyl bromide were found to be identical, small variations in these peaks could affect the 5:2 ratios. Thus, the HMT method could be quantitatively more correct, whereas this \(^{2}H\)-NMR method would be most useful for evaluating relative changes in the fractional enrichments. Nevertheless, both estimates are in the range of 50–65% observed after overnight fasting (2,6,16,18,24).

Interestingly, the 3:2 ratios gave lower (\( P = 0.029 \)) estimates of gluconeogenesis than the 5:2 ratios, which is similar to findings in fasted rats (11). The differences between the 6:2 and 5:2 ratios were even greater (19, \( P < 0.001 \)) and similar to that reported for the HMT method (16–22% at 14–22 h of fasting) (9,10). The 6:2 ratio has been used for measuring gluconeogenesis (9) but gives an underestimation because 1) binding of \(^{2}H\) at position 6 depends on the exchange of the hydrogens of pyruvate and alanine and of oxaloacetate and fumarate with those of water in their equilibrations before oxaloacetate’s conversion to glucose, and 2) the conversion of glycerol to glucose does not result in binding of \(^{2}H\) at position 6 (25).

Assuming complete exchange of hydrogens in body water at the level of pyruvate (pyruvate alanine and oxaloacetate fumarate), the 6:2 ratios reflect gluconeogenesis from the level of PEP, and the difference of 5:2 and 6:2 ratios reflects gluconeogenesis from glyceral. Using the individual data at each time point, gluconeogenesis from glyceral contributes to EGP by 27 ± 4 and 19 ± 4% in type 2 diabetic patients and control subjects (\( P = 0.05 \)), respectively. The estimates of the contribution of glycerol are somewhat higher than in previous reports (13,25). Nevertheless, in type 2 diabetic patients, the lower contribution of glycogen to EGP could be due to augmented gluconeogenesis from glycerol rather than from PEP, which was similar in type 2 diabetic patients and control subjects, as demonstrated by comparable 6:2 ratios (Table 1).

In contrast, the 1:2 ratio would have overestimated the contribution of gluconeogenesis to EGP, which is in
agreement with a previous study (25). That is presumably explained by a contribution of glycogenolysis to the $^2\text{H}$ at position 1 during short-term fasting because of equilibration of mannose-6-P with glucose-6-P via fructose-6-P (25).

To our knowledge, 4:2 ratios have not yet been compared with 5:2 ratios in humans in a similar experimental setup, i.e., after $^2\text{H}_2\text{O}$ ingestion. The 4:2 ratios were higher than the 5:2 ratios in both healthy and type 2 diabetic subjects, suggesting that this finding could be a constant phenomenon. With the equilibration at the triose-P level, one might expect that labeling at position 3 and 4 would approach that at position 5 but not exceed it.

Current limitations such as the large sample volume and long duration of spectra accumulation should be reduced by employing a custom-built NMR probe. With such equipment the costs of our method will be in the range of those of the HMT method but much less compared with in vivo $^{13}\text{C}$ MRS.

Our study demonstrates that with all methods used, the contribution of gluconeogenesis to EGP is also higher in well-controlled type 2 diabetic patients as previously demonstrated for type 2 diabetic patients with moderate metabolic control by $^{13}\text{C}$-MRS (1,27) and the HMT method (24,28). Likewise, EGP was elevated in type 2 diabetic patients, which is in line with the majority of studies (29,30). As glycogenolysis was decreased in type 2 diabetic patients, probably due to lower hepatic glycogen concentrations, augmented gluconeogenesis mainly explains the increased EGP in these patients.

The conversion of galactose to glucose serves as a surrogate for glycogenolysis, since the same pathway is followed in both processes, i.e., the galactose-1-P formed is converted to glucose-1-P and hence to glucose-6-P. The decline in the percent of contribution of gluconeogenesis to glucose production was the same, ~30% over the 4 h of galactose infusion, confirmed by either the HMT or $^2\text{H}$-NMR method. Of the approximate $11.4 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ infused, ~29%, i.e., $3.1 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, appears to have been deposited in liver glycogen and ~23% (fractional decrease in gluconeogenesis times EGP), i.e., $2.6 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, was converted to glucose that entered the circulation. While the use of 5:2 ratios gives a measure of

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

**FIG. 4.** Plasma glucose concentrations (A) ($*P < 0.05$ vs. 40 h), liver glycogen concentrations (B) ($*P < 0.01$ vs. 40 h), and percent gluconeogenesis obtained from in vitro $^2\text{H}$-NMR (C) ($*P = 0.02$ vs. 40 h) during infusion of galactose (9 g/h) in healthy volunteers ($n = 3$).
the fractional contribution of gluconeogenesis to glucose production, the increase in glycogen content cannot be taken as a measure of the contribution of gluconeogenesis to glycogen formation under these conditions (31), since the conversion of galactose to glycogen does not require glucose 6-P as an intermediate, i.e., galactose-1-P is converted to UDP-glucose and hence glycogen. Also, galactose may not be considered a gluconeogenic substrate, since its conversion to glucose requires only epimerization of its hydroxyl at position, while its carbon skeleton remains intact.

In conclusion, in vitro 2H-NMR offers an alternative valid approach to directly quantify gluconeogenesis because this method 1) correlates linearly with established methods such as the HMT method and in vivo 13C MRS, 2) can be effectively applied in type 2 diabetic patients, and 3) allows monitoring of rapid changes in glycogenolysis and gluconeogenesis in humans. This could be of clinical relevance for studying glucose metabolism and new therapeutic strategies for diabetes treatment.

ACKNOWLEDGMENTS

The studies were supported by the Austrian Science Foundation (P13219-MOB, P13722-MED to M.R., P13718-CHE to E.H.), Novo Nordisk (to W.W.), National Institutes of Health (RO1-DK-14507 to B.R.L.), Austrian National Bank (9127 to H.S. and M.R.), and the Austrian Academy of Sciences (427/1997 to H.S.).

We thank D. Weghuber and the lab of the Division of Endocrinology and Metabolism, University of Vienna, for technical assistance and E. Moser, PhD, and the Department of Radiology for support.

REFERENCES