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Quantifying the contribution of gluconeogenesis to glucose production in fasted human subjects using stable isotopes

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The contribution of gluconeogenesis to glucose production is estimated from the enrichment of the H bound to C-5 of glucose relative to either that bound to C-2 of glucose or the enrichment in body water on ingesting ^2H_2O in the fasted state. Contributions of all gluconeogenic substrates are included in the estimate and the limitation of an uncertain precursor enrichment removed. The half-life of ^2H_2O in body water precludes a repeat study for many weeks. Glycogen cycling could result in underestimation, but there is evidence that glycogen cycling does not occur in liver in the fasted state. Gluconeogenesis has been estimated by mass-isotopomer-distribution analyses, usually by administering ^{13}C-labelled glycerol. Underestimates emphasize the major limitation of the method, i.e. the need to assume a single enrichment of the precursor pool. Estimates of gluconeogenesis from isotopomer distribution in arterial-blood glucose and lactate on infusing [U-$^{13}C_6$] glucose are unreliable, as a proportion of the glucose is formed from glycerol and from amino acids not converted to glucose via pyruvate. Loss of label in the Krebs cycle and relying on enrichment of arterial-blood lactate as a measure of hepatic pyruvate further add to the uncertainty. Estimates of the rate of gluconeogenesis by NMR are obtained by subtraction of the rate of glycogenolysis determined by NMR from the rate of glucose production. Estimates are then the mean rate for the period over which glycogen contents are measured. Technical considerations can limit the accuracy of analyses and result in overestimates.

^{2}H-labelled water: Gluconeogenesis: Stable isotopes: NMR: Mass-isotopomer analysis

An estimate of the contribution of gluconeogenesis (GNG) to glucose production in the fasted state requires an estimate of glucose production. Production can be estimated by measuring: (1) blood glucose concentrations across liver and kidney together with blood flows through those organs (the balance method; Bondy *et al*. 1949) or (2) enrichments of blood glucose on infusing stable-isotope-labelled glucose, or specific activities of blood glucose on infusing a radioactively-labelled glucose (the kinetic method; Hetenyi *et al*. 1983). Estimates obtained by the balance method are underestimates, as a proportion of the glucose is utilized by liver and kidney. The latter can be accounted for by measuring label taken up by those organs when labelled glucose is infused (DeFronzo *et al*. 1983, 1985). The kinetic method gives underestimates, as a proportion of the label from the infused glucose is incorporated into the glucose produced (recycling). No ^3H from [3-^{3}H]glucose and only a small amount from [6-^{3}H]glucose is recycled (Landau, 1993). ^3H from [3-^{3}H]glucose can be removed in triose phosphate cycling (glucose → triose phosphate → glucose), resulting in an overestimation of production, but the extent is small. Use of [6,6-$^2H_2$]glucose is close to ideal, since

Abbreviations: DHAP, dihydroxyacetone-3-phosphate; F6P, fructose-6-phosphate; GAP, glyceraldehyde-3-phosphate; G6P, glucose-6-phosphate; GNG, gluconeogenesis; HMT, hexamethylenetetramine; MIDA, mass-isotopomer-distribution analysis.

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radioactivity is avoided and recycling is negligible. [U-$^{13}C_6$]- Glucose is an alternative, but costs more.

Glucose is produced in the fasted state by GNG and glycogenolysis. Estimates of the contribution of GNG have generally been made by two methods. In the first method concentrations across the liver of blood lactate, pyruvate, glycerol and GNG amino acids, as well as glucose, together with blood flow, are measured. The fractional contribution of GNG to glucose production is then calculated, assuming all the GNG substrates taken up are converted to glucose (Wahren *et al*. 1977). Thus, estimates are maximal, except that a proportion of GNG substrates are produced and converted to glucose within the liver. Since access to the portal vein is usually not feasible in human subjects, measurements are made across the splanchnic bed. After an overnight fast, in most reports, little if any difference in glucose concentration across the kidney is detected (Bjorkman *et al*. 1980). Based on measurements of uptake across the kidney of label from labelled glucose, kidney has been concluded to contribute as much as 30 % to glucose production (Stumvoll *et al*. 1995), but that estimate is in doubt (Ekberg *et al*. 1999). In the second method, a ^{14}C- or ^{13}C-labelled GNG substrate is infused and at steady-state the specific activity or enrichment in blood glucose is compared with that in the circulating substrate (Chaisson *et al*. 1977). Underestimates are obtained because only one of the several substrates converted to glucose is measured and because any substrate converted to glucose via pyruvate has oxaloacetate as an intermediate. Oxaloacetate is also an intermediate in the Krebs cycle, so that in the process of GNG unlabelled C is exchanged for the labelled C.

The focus of the present paper is on four methods recently introduced for quantifying the contribution of GNG to glucose production using stable isotopes i.e. heavy water, mass-isotopomer-distribution analysis (MIDA), [U-^{13}C]glucose isotopomers, and ^{13}C NMR spectroscopy.

Methods and results

Heavy water

During a fast, ^2H_2O is ingested to reach a body water enrichment of 0·25–0·5 % (Landau *et al*. 1995*c*, 1996; Chandramouli *et al*. 1997; Landau, 1997). Ingestion of a single dose to achieve 0·5 % enrichment can cause vertigo, therefore the dose is given over 2–4 h. When the enrichment at C-5 in blood glucose becomes constant, within a few hours after normal subjects ingest ^2H_2O, i.e. there is steady-state, then:

$$\text{fractional contribution of GNG} = \frac{^2\text{H enrichment at C-5}}{^2\text{H enrichment in body water}}.$$

This relationship is based on a H from body water being bound to C-5 of glucose formed by GNG from all GNG substrates and none being bound to C-5 of glucose formed by glycogenolysis, i.e. pyruvate → phosphoenolpyruvate $\xrightarrow{^2H_2O}$ [2-^{2}H]glycerate-2-phosphate → [2-^{2}H]glyceraldehyde-3-phosphate (GAP); glycerol → dihydroxyacetone-3-phosphate (DHAP) $\xrightarrow{^2H_2O}$ [2-H] GAP, and DHAP + [2-^{2}H]GAP → [5-^{2}H]glucose.

By the time of the last ^2H_2O ingestion or only about 1 h later:

$$\text{fractional contribution of GNG} = \frac{^2\text{H enrichment at C-5}}{^2\text{H enrichment at C-2}}.$$

This relationship is based on a H from body water being bound to C-2 of glucose during both GNG and glycogenolysis, i.e. fructose-6-phosphate (F6P) $\xrightarrow{^2H_2O}$ [2-^{2}H]glucose-6-phosphate (G6P) → [2-^{2}H]glucose and glycogen → glucose-1-phosphate → G6P → F6P $\xrightarrow{^2H_2O}$ [2-^{2}H]G6P → [2-^{2}H]glucose. For the equation to be valid there must be essentially complete isotopic equilibration between G6P and F6P. As evidence for equilibration, when hepatocytes were incubated with lactate and pyruvate in a medium containing ^3H_2O (Rognstad *et al*. 1974), the radioactivity of the H bound to C-2 of the glucose formed was 100 (SE 2) % (*n* 6) of the radioactivity in the water-H. When normal subjects were fasted overnight and long term, enrichment at C-2 of glucose was approximately equal to the enrichment in body water (Landau *et al*. 1996; Chandramouli *et al*. 1997). This relationship was also found in patients with malaria (Dekker *et al*. 1997*b*) and cirrhosis (Petersen *et al*. 1999) who were fasted overnight and in subjects in whom the effect of fatty acid elevation on GNG was examined (Chen *et al*. 1999).

Enrichment at C-5 of a portion of blood glucose is determined chemically by converting the glucose to xylose with the removal of C-6 (Landau *et al*. 1996). The xylose is then oxidized with periodate to yield formaldehyde containing C-5 with its H. Condensation of six molecules of the formaldehyde with four molecules of NH_3 yields one molecule of hexamethylenetetramine (HMT). All H in HMT are derived from the formaldehyde, and HMT can be assayed directly using a mass spectrometer. Its molecular mass is 140 (m + 0). The percentage of molecules with one ^{2}H (m + 1) is determined. C-1 is removed enzymically from another portion of the blood glucose to form ribulose-5-phosphate which is reduced to the polyol phosphates, ribitol-5-phosphate and arabitol-5-phosphate. Oxidized with periodate, these compounds yield formaldehyde and hence an HMT containing the H bound to C-2. The six-fold magnification in assaying the enrichment of the H attained by using HMT is critical in quantifying GNG while giving a safe well-tolerated dose of ^2H_2O. Enrichment in body water is determined for samples of plasma or urine by isotope-ratio mass spectrometry.

To measure glucose production, an infusion of [6,6-$^2H_2$]glucose with a primer is given together with the ^2H_2O (Chandramouli *et al*. 1997). A portion of the blood glucose, when steady-state is reached (usually by 3 h), is oxidized to yield C-6 with its two H in formaldehyde and hence in HMT. The percentage of the HMT molecules having two ^{2}H bound to C-6 is determined, i.e. the

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percentage of the molecules of molecular mass 142 (m + 2). Then:

$$\text{glucose production} = \frac{[6,6\text{-}^2H_2]\text{glucose (infusion rate} \times \text{enrichment)}}{\text{enrichment (m+2) at blood glucose C-6}} - [6,6\text{-}^2H_2]\text{glucose infusion rate.}$$

The fractional contribution of GNG to glucose production multiplied by glucose production equals the amount of glucose formed by GNG. Administration of ^2H_2O does not affect the m + 2 enrichment at C-6 or [6,6-$^2H_2$]glucose administration the m + 1 enrichments at C-2 and C-5 of blood glucose.

The method measures the sum of the contributions of GNG to glucose production by liver and kidney. Using the enrichment at C-5 : enrichment in water there are only a few potential errors. As a proportion of the G6P formed by glycogenolysis is converted to triose phosphates that are reconverted to G6P which is then hydrolyzed to glucose, i.e. triose phosphate cycling, GNG is overestimated. In normal subjects, after an overnight fast, triose phosphate cycling, measured by the amount of ^3H from [1-^{3}H]galactose bound to C-6 of blood glucose, causes an overestimation of GNG of only about 3 % (Chandramouli *et al.* 1999). Pentose-cycle activity, transaldolase (*EC* 2.2.1.2) in the non-oxidative portion of the cycle, and transaldolase-catalysed exchange between F6P and GAP could also result in an overestimation of GNG. Pentose-cycle activity probably also causes an overestimation of only a few percentage units (Magnusson *et al*. 1988, 1990). The contribution of the transaldolase reactions is likely to be small, but has yet to be quantified.

When equilibration of G6P with F6P is incomplete, GNG would be overestimated using the ^2H enrichment at C-5 : enrichment at C-2. Again, however, under conditions thus far studied, it is essentially complete, i.e. ^2H enrichment at C-2 is approximately equal to ^2H_2O at steady-state. From the retention of 20 % ^3H in the conversion of [2-^{3}H]galactose to glucose (Wajngot *et al*. 1989), it might be expected to be only 80 % complete (Landau *et al*. 1995*c*), but the 20 % retention is attributable to an isotope effect resulting in the incomplete removal of ^3H of [2-^{3}H]G6P (Katz *et al*. 1978). Direct release of glucose from hydrolysis of 1,6-glucosyl branches in glycogen would also result in an overestimate of GNG. This overestimation could amount to about 4 % in the subject fasted overnight, but could be reduced by glucose cycling, which labels the H at C-2 of the glucose (Landau *et al*. 1996). The finding that ^2H enrichment at C-2 is approximately equal to ^2H_2O indicates that the contribution from hydrolysis is very small.

Glycogen cycling would result in the deposition in glycogen of [2,5-^{2}H]G6P formed by GNG, while that amount of [2-^{2}H]glucose would be released by glycogen breakdown. Hence, GNG would be underestimated. However, there is good evidence that glycogen cycling does not occur in the fasted state, at least in normal subjects (less than 2 % of the rate of glucose production) (Petersen *et al*. 1998). On the otherhand, it has been stated that glycogen cycling occurs in the fasted state in normal subjects (Fried *et al*. 1996; Katz & Tayek, 1998). However, reference was made to measurements made by Magnusson *et al*. (1994) while glucose was infused to maintain a plasma glucose level of 1·70 g/l for several hours in subjects who had been fasted. Evidence that glycogen cycling does not occur in fasted diabetics comes from studies in which no label was found in liver glycogen when labelled glucose was given to fasted alloxan-induced and streptozotocin-induced diabetic rats (Freedmann *et al*. 1963; Wi *et al*. 1996). The major limitation of the method is the long half-life of ^2H_2O in body water, which prohibits a repeat study in the same subject for many weeks (Chandramouli *et al*. 1997). Since the contribution of GNG increases with fasting by about 1·5 %/h (Chandramouli *et al*. 1997), values based on analysis of a blood glucose sample are underestimated by a few percentage units. The reason for this underestimation is that the glucose in the blood is formed not only at the time of sampling, but also in the preceding period, although in smaller amounts. A similar consideration applies to the estimate of glucose production, since production slowly decreases during fasting.

The fractional contribution of GNG to glucose production can also be estimated from the enrichment (m+1) of the H bound to C-6 of glucose on ingesting ^2H_2O, using either the enrichment at C-6 : enrichment at C-2, or at steady-state the C-6 enrichment : water enrichment (Landau *et al*. 1995*b*). However, [6,6-$^2H_2$]glucose cannot then be used to estimate glucose production. Furthermore, GNG is underestimated, since (1) enrichment at C-6 is less than that in body water, as isotopic equilibrations of pyruvate with alanine and oxaloacetate with fumarate (resulting in the enrichment at C-6) are incomplete, and (2) GNG from glycerol is not included in the estimate.

Using the heavy-water method, the contribution of GNG to glucose production has been estimated at 47 % after 14 h, 67 % after 22 h and 92 % after 42 h of fasting by normal subjects (Landau *et al*. 1996). Between 14 and 22 h, while the contribution of GNG increased, glucose production declined, so that the contribution of GNG remained unchanged (Chandramouli *et al*. 1997). There appears to be a small increase above normal in the contribution of GNG in subjects with non-insulin-dependent diabetes mellitus (Wajmgot *et al*. 1999). However, glucose production, while normal or near normal in mild hyperglycaemia (fasting plasma glucose concentrations 8-10 mM/l) increases with more acute hyperglycaemia (Hother-Nielsen & Beck-Nielsen, 1990; Jeng *et al*. 1994).

Mass-isotopomer-distribution analysis

A labelled GNG substrate is administered and the mass-isotopomer distribution in blood glucose determined (Neese *et al*. 1995). For example, if [U-$^{13}C_3$]glycerol is infused, DHAP and GAP will have the same proportion of molecules with three ^{13}C (i.e. molecular mass 93) and with no ^{13}C (i.e. molecular mass 90) because of their extensive equilibration. They will form glucose with no ^{13}C (molecular mass 180) with three ^{13}C (molecular mass 183) and with six ^{13}C (molecular mass 186). In the calculations M with a subscript

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represents the proportion of the molecules of glucose having the number of ^{13}C in the subscript; correspondingly m with a subscript represents the proportion of the molecules of DHAP and GAP having the number of ^{13}C atoms in the subscript. Assuming m_3 for DHAP and GAP (designated x) is 0·1, i.e. ten of 100 molecules of GAP and DHAP each have three ^{13}C. Of 100 molecules glucose formed from 200 molecules GAP and DHAP, then M_6 will be $x^2 = 0{\cdot}01$ and M_3 will be 2 (x–x^2) = 0·18; M_3:M_6 will be 0·18/0·01 = 18. Assuming instead of 100 % glucose being formed from triose phosphates, only 50 % is formed by GNG, and hence 50 % by glycogenolysis, then $M_6 = 0{\cdot}005$ and $M_3 = 0{\cdot}09$. The general expressions are $M_6 = x^2/y$ and $M_3 = 2(x–x^2)/y$, where y is the extent of dilution by glycogenolysis of the labelled glucose formed by GNG: in the example y=2. Since there are two equations and two unknowns (x and y), if M_3 and M_6 in blood glucose are determined, the contribution of GNG relative to glycogenolysis can be estimated. If a single labelled ^{13}C substrate is infused (e.g. [2-^{13}C]glycerol), M_1 and M_2 replace M_3 and M_6. The equations are simplified. More complex expressions exist, for example to take into account formation from [U-$^{13}C_3$]glycerol of DHAP and GAP as isotopomers with one and two ^{13}C atoms, and their condensation. In most studies [U-$^{13}C_3$]glycerol (Landau *et al*. 1995*a*) or [2-^{13}C]glycerol (Hellerstein *et al*. 1997) has been used; [U-$^{13}C_3$]lactate has also been used (Lee *et al*. 1994).

The major assumption in applying MIDA is that there is a single pool of the precursor, i.e. in the example described earlier the triose phosphate pool is uniform in containing 10 % of the molecules with three ^{13}C. However, uptake of glycerol is almost complete in its single passage through the liver. Hepatocytes closer to the portal-vein area of the liver lobule are therefore exposed to much higher concentrations of the glycerol than hepatocytes in the hepatic-vein area, relative to other GNG substrates (Landau *et al*. 1995*b*; Previs *et al*. 1995). Thus the decrease in lactate concentration along the lobule is not as great. As a result, when using ^{13}C-labelled glycerol under standard conditions, the contribution of GNG is underestimated by MIDA. This underestimation can be explained by considering the cells closest to the hepatic vein, because of a lack of ^{13}C-glycerol, synthesize essentially unlabelled glucose. The MIDA method cannot differentiate between this unlabelled glucose synthesized by GNG, and glucose released by glycogenolysis.

In accordance with the absence of a single precursor pool, when [U-$^{13}C_3$]glycerol was infused for 5 h into healthy subjects fasted for 60 h the contribution of GNG to glucose production was estimated to be only 60 % (Landau *et al*. 1995*b*), and only 78 % when infused with [2-^{13}C]glycerol for 4 h (Hellerstein *et al*. 1997), rather than > 90 % expected with glycogen depletion. The greater percentage contribution with [2-^{13}C]glycerol when compared with [U-$^{13}C_3$]glycerol can be explained by the need to infuse more [2-^{13}C]glycerol, resulting in a smaller concentration gradient along the liver lobule. More [2-^{13}C]glycerol must be infused because of 'background' in the analysis, i.e. the natural abundance of glucose molecules with one and two ^{13}C is much greater than that glucose with three and six ^{13}C. Acetaminophen has also been given to subjects receiving [2-^{13}C]glycerol in order to form acetaminophen glucuronide and this factor may also contribute to a decrease in the concentration gradient of the glycerol along the liver lobule. The reason is that the acetaminophen, while not reported in the publications, was dissolved in 50 g D-glucose/l in water –propylene glycol–ethanol (5:4:1, by vol.; Ameer *et al*. 1983; Siler *et al*. 1998). Propylene glycol is metabolized to lactate and ethanol is metabolized to acetic acid with generation of NADH. The utilization of glycerol is inhibited by increased NADH : NAD (Lundquist *et al*. 1965).

Hellerstein and co-workers (Dekker *et al*. 1997*a*; Hellerstein *et al*. 1997) have attributed the lower contribution of GNG obtained using [U-$^{13}C_3$]glycerol (60 % *v*. 78 %) to analytical error. As evidence they have claimed that when liver was perfused with [U-$^{13}C_3$]glycerol (Previs *et al*. 1995), the contribution of GNG ranged from 75 to 92 % due to assay variability. However, the GNG contribution was 75 % when the level of perfusion was 0·1 mM-glycerol, 85 % at 0·5 mM-glycerol, and 92 % at 1·5 mM-glycerol, i.e. the contribution increased with increasing level of glycerol administered (also in agreement with data from Peroni *et al*. 1995). As a result of the claim of Hellerstein and co-workers (Dekker *et al*. 1997*a*; Hellerstein *et al*. 1997) of a weakness in the assays using [U-$^{13}C_3$]glycerol, Previs *et al*. (1998) compared measurements obtained by MIDA using [U-$^{13}C_3$]glycerol and [2-^{13}C]glycerol. They found no difference. Other experiments show similar underestimates using MIDA (Previs *et al*. 1998).

Just as with the ^2H_2O method, values for the contribution of GNG obtained by MIDA are overestimated by the extent to which G6P derived from glycogen (before its conversion to glucose) undergoes triose phosphate cycling, the pentose cycle, and transaldolase reactions. Hellerstein *et al*. (1997) attribute their lower-than-expected estimates of the contribution of GNG to the occurrence of glycogen cycling in the fasting state rather than a concentration gradient across the liver lobule, but their evidence for glycogen cycling is poor. The evidence is based first on an estimate of glycogen synthesis, assessed using [1-^2H]galactose and glucuronide formation after long-term (60–70 h) fasting. For their estimate they presuppose that the reaction glucose-1-phosphate + uridine triphosphate → uridine diphosphate glucose + pyrophosphate has been established biochemically to be irreversible, when biochemical evidence is to the contrary (Guynn *et al*. 1974; Newsholme & Leech, 1985; Davidson *et al*. 1988; Chandramouli *et al*. 1997). They also conclude that the hepatic glycogen content after 60–70 h fasting is sufficient for such levels of glycogen cycling to occur, despite the extent to which the last glycogen deposited is the first released. They also reason that there must be significant conversion of the [1-^2H]galactose to glycogen, since only a minor proportion of ^2H is recovered in glucose. However, galactose is converted to G6P which has fates other than conversion to glucose. In addition, in the conversion of the galactose to glucose, ^2H appears to be lost in the reactions G6P ↔ F6P ↔ mannose-6-phosphate (Chandramouli *et al*. 1999). Further evidence of Hellerstein *et al*. (1997) includes a release of ^2H-labelled glucose on glucagon administration. However, this release occurred after infusion over 10 h of approximately 3·5 g galactose,

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2·2 g glucose, 9 g glycerol, 21 g propylene glycol (equivalent to 25 g lactic acid) and 4 g ethanol, so their subjects were questionably in the fasting state.

Hellerstein *et al*. (1997) also conclude that glycogen cycling occurred because after the 10 h of infusion the contribution of GNG calculated by MIDA was 84–96 %, compared with the 78 % after 4 h of infusion. They imply this means replacement over time of the unlabelled glucosyl units of glycogen by labelled units formed via GNG, i.e. glycogen cycling. However, the concentration gradient of labelled glycerol along the liver lobule could dissipate with the quantities of propylene glycol and ethanol infused. In contrast, Chandramouli *et al*. (1997) observed the same contribution by GNG whether determined a few hours or many hours after ^2H_2O ingestion. Of course, ethanol and propylene glycol were not given. Magnusson *et al*. (1987) reported that glucuronide formation could be used to represent the hepatic uridine diphosphate–glucose pool that is the precursor of glycogen formation, but Hellerstein *et al*. (1986) reported that the glucuronide could not be used because it was representative of a different pool. Re-interpretation of the data of Hellerstein *et al*. (1986) supported the conclusion of Magnusson *et al*. (1987). The glucuronide has been used to trace glycogen formation since that time.

The contribution of GNG determined using [2-^{13}C]-glycerol has been estimated to be approximately 35 % in normal subjects fasted overnight (Hellerstein *et al*. 1995, 1997) and 33 % in subjects with non-insulin-dependent diabetes mellitus (Christiansen *et al*. 1995). In two subjects infused with [U-$^{13}C_3$]lactate, after an overnight fast the contribution of GNG was 39 and 50 %, and 70 % after 24 h of fasting (Lee *et al*. 1994).

[U-$^{13}C_6$]glucose isotopomer

Tayek & Katz (1996, 1997) introduced a method for estimating the contribution of GNG to glucose production by infusing [U-$^{13}C_6$]glucose. In the calculation M with a subscript represents the percentage of the molecules of glucose having the number of ^{13}C atoms in the subscript; correspondingly, m with a subscript represents the percentage of the molecules of lactate having the number of ^{13}C in the subscript. Glucose production is estimated at steady-state from M_6 in blood glucose and the rate of infusion of the [U-$^{13}C_6$]glucose and its M_6. The equation introduced by Landau *et al*. (1998) for relating the fractional contribution of GNG to the isotopomer distribution in arterial-blood glucose and lactate is:

$$\text{fractional contribution of GNG} = \frac{M_1 + M_2 + M_3}{2(m_1 + m_2 + m_3)} = \frac{0.5(M_1 + M_2 + M_3)}{m_1 + m_2 + m_3}. \quad (1)$$

It is assumed that all glucose is produced by GNG and 2 % of the lactate is formed from the [U-$^{13}C_6$]glucose, i.e. four of every 200 molecules have three ^{13}C. Those 200 molecules, if converted without loss of ^{13}C, would form 100 molecules glucose, four of which would have three ^{13}C. Hence, the fractional contribution of GNG to glucose production would be $M_3/2m_3$ = 4 %/2(2 %) = 1·0, i.e. 100 % contribution of GNG. The likelihood of a glucose molecule being formed with six ^{13}C is negligible, i.e. $(0{\cdot}02)^2 = 0{\cdot}0004$, i.e. only 1 in 2500 molecules.

In actuality, at steady-state, the labelled molecules of arterial lactate will have three, two or one atoms of ^{13}C. Furthermore, in the conversion of the lactate to triose phosphate in the process of GNG, ^{13}C will exchange with ^{12}C in the Krebs cycle. As an example:

total molecules:	200 lactate →	200 triose phosphate →	100 glucose
no. with three ^{13}C:	2 (m_3 1 %)	0 (m_3 0 %)	0 (M_3 0 %)
no. with two ^{13}C:	1 (m_2 0·5 %)	2 (m_2 1 %)	2 (M_2 2 %))
no. with one ^{13}C:	1 (m_1 0·5 %)	2 (m_1 1 %)	2 (M_1 2 %).

The proportion of glucose produced by GNG is again: $(M_1 + M_2 + M_3)/2(m_1 + m_2 + m_3) = (2 + 2 + 0)/2(0{\cdot}5 + 0{\cdot}5 + 1) = 1{\cdot}0$. M_1, M_2 and M_3 would be lowered by the extent to which glycogenolysis contributed to glucose production. Thus, if GNG and glycogenolysis contributed equally to glucose production, M_1, M_2 and M_3 would be halved, i.e. their sum would be 1 % in the example, and the fractional contribution of GNG would be 0·5. Glucose molecules with one ^{13}C can also be formed by the fixing by pyruvate of $^{13}CO_2$ formed from the [U-$^{13}C_6$]glucose. Correction can be made for that amount (Landau *et al*. 1998).

Thus, the equation for calculations of the contribution of GNG using mass isotopomers is analogous to the method where a radioactive substrate is given and the specific activity of the glucose is compared with that in the circulating substrate. For example, if [^{14}C]lactate 50 disintegrations/min per μmol, was infused and the specific activity of the glucose was found to be 100 disintegrations/min per μmol, GNG would be 100 %, i.e. glucose/2 (lactate) = 100/2 (50) = 1·0. However, ^{14}C of lactate is exchanged for ^{12}C in the process of GNG to an unknown extent, so that the proportion despite 100 % contribution of GNG will be less then 1·0. Using isotopomers the extent of exchange is known. If a molecule of lactate has one or two rather than three ^{13}C because of exchange, had there been no exchange the molecule would have yielded a glucose molecule with three atoms of ^{13}C. Thus, by giving the same weight in the equation to labelled molecules irrespective of their number of ^{13}C, the effect of exchange is removed.

Values for the contribution of GNG calculated by equation 1, using isotopic distributions found in subjects fasted overnight and long term, are about half those determined by the ^2H_2O method. Several substances contribute to the underestimations (Landau *et al*. 1998). Only substrates converted to glucose via pyruvate are included in the estimates. Glucose formation from glycerol and amino acids (e.g. aspartate, glutamate and glutamine) is included in the estimates as a contribution of glycogenolysis. Also, label will be lost, and hence the glucose molecules formed will be estimated as being derived from glycogenolysis rather than GNG, to the extent that (1) exchange is complete, so that a molecule of lactate labelled with ^{13}C forms glucose without ^{13}C and (2) there is exchange between labelled intermediates in the Krebs cycle with unlabelled amino acids in the circulation, i.e. glutamine ↔ glutamate ↔ α-ketoglutarate and aspartate ↔

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oxaloacetate. Also, in this method the enrichment in arterial lactate is assumed to be the same as that of hepatic pyruvate. While these enrichment levels may be similar, the enrichment in hepatic pyruvate is likely to be lower. A factor F can be introduced to take account of these contributions to the underestimation (Landau, 1999):

$$\text{fractional contribution of GNG} = F\,(0{\cdot}5(M_1 + M_2 + M_3)/(m_1 + m_2 + m_3)). \quad (2)$$

Unfortunately the value of F cannot be predicted for any condition nor can its variability from condition to condition. The following example emphasizes the uncertainty. Suppose two groups are compared, and from isotopomer distributions the contribution of GNG calculated using equation 1 is 40 %. If F is 1·5 for the first group and 2·5 for the second group, then by equation 2 the actual contribution of GNG for the first group is $1{\cdot}5 \times 40 = 60$ % and for the second group is $2{\cdot}5 \times 40 = 100$ %.

Glycogen cycling, if it occurred, would also result in an underestimation of GNG by this method. Pentose-cycle activity would only result in an underestimate by the extent to which ^{13}C was completely replaced by ^{12}C. Neither triose phosphate cycling nor transaldolase exchanges would alter estimates.

The equation proposed by Tayek & Katz (1997) to calculate the contribution of GNG uses weighted isotopomers, i.e. calculation is based on labelled atoms not molecules. Most importantly in their equation, the factor 0·5 is not included. Similarly, 10 years ago Katz *et al*. (1989) omitted the factor 0·5 when estimating (using [U-$^{13}C_6$]glucose) the indirect pathway of glycogen formation, i.e. glucose → lactate → glycogen. That omission was noted by Des Rosiers *et al*. (1990). Katz *et al*. (1991*a*,*b*) then corrected their equations. Katz & Tayek (1998) claimed validity for their equation because when applying it to isotopomer distributions in subjects fasted overnight and long term, the contributions of GNG to glucose production were calculated to be 41 and 92 % respectively, in accord with estimates obtained by the ^2H_2O method. Since $2 \times 0{\cdot}5 = 1$ they are in essence, without recognizing it, including the factor 0·5, but fixing F at a value of 2. Since F is approximately equal to 2 under their conditions they obtain reasonable estimates. Estimates by Wykes *et al*. (1998), applying the equation of Tayek & Katz (1997) to isotopomer distributions obtained by [U-$^{13}C_6$] glucose administration to fasted piglets, exceeded 100 % in accord with an F value of less than 2.

The proportion of glucose that is recycled to glucose, also called Cori cycling (Cori, 1931; i.e. glucose → lactate → glucose), can be estimated from the isotopomer distribution in glucose:

$$\text{glucose recycled} = \frac{0.5(M_1 + M_2 + M_3)}{0.5(M_1 + M_2 + M_3) + M_6} \quad (3)$$

Again, the equation used by Tayek & Katz (1997) is incorrect because it lacks the factor 0·5. The equation proposed by Kalderon *et al*. (1989) was also noted by Des Rosiers *et al*. (1990) to lack this factor. Cycling, calculated using equation 3, is underestimated by the extent to which labelled molecules of lactate lose all their label by exchange before their conversion to glucose and there is exchange between labelled intermediates in the Krebs cycle and unlabelled amino acids in the circulation (Landau, 1999).

^{13}C NMR spectroscopy

^{13}C NMR spectroscopy is an elegant method for measuring glycogen content and hence net glycogen changes. Using the method to estimate the contribution of GNG to glucose production, the rate of glycogen disappearance during fasting is calculated from the difference in glycogen concentration at two time points that are distant from each other multiplied by liver volume. Subtracting the rate of glycogen disappearance from the rate of glucose production then yields the estimated rate of GNG. In the procedure the subject is brought to the instrument's magnet several hours after ingesting a liquid meal. The contribution of GNG is then estimated over a period of fasting by measuring glycogen concentration several times. Liver volume is measured and the subject is transferred from the magnet area, usually at the end of the fast, to measure glucose production.

In the first reported study (Rothman *et al*. 1991), subjects entered the magnet at 22·00 hours, 4 h after the meal, and glucose production was measured 22 h post-prandially. It was concluded that glycogenolysis proceeded at a constant rate over the 22 h of fasting. Subtracting the contribution of glycogenolysis from the measured glucose production gave an overall contribution of GNG over the 18 h which was estimated at 64 % of production. If glucose production was greater during initial period of fasting, the contribution of GNG would be higher. Balance studies do not show sufficient uptake of substrate by the splanchnic bed to account for 64 % production (Wahren *et al*. 1977).

In a second study (Petersen *et al*. 1996), normal subjects were fed on a meal containing 150 g glucose, and then placed in the instrument's magnet between 5–6 h and 10-12 h of fasting. It was concluded that glycogen disappearance was linear between 6 and 12 h. There were no measurements between 6·5 and 10 h. The subjects were instead transported to a clinical centre so that glucose production could be measured between 7 and 9 h by infusing [6,6-$^2H_2$]glucose. The contribution of GNG was estimated to be 55 % (*n* 13) over the period 6–12 h, varying from 24 to 90 %. In a third study (Magnusson *et al*. 1992) glycogen levels in normal and diabetic subjects were measured between 4 and 23 h, with glucose production determined at 22 h. Subjects ingested a meal containing 90 g carbohydrates. The contribution of GNG over the 23 h period was calculated to be 70 % in the normal subjects and 88 % in the diabetic subjects. If the mean contribution of GNG for the normal subjects over the first 12 h was approximately 50 % (Petersen *et al*. 1996), in order to reach a mean level of 70 % for the whole period, the contribution of GNG during the last 12 h must have averaged approximately 90 %. If the mean contribution of GNG for diabetic subjects over the first 12 h was 60 %, it must have been more than 100 % over the last 11 h. If the contribution of GNG increases with time, in order to reach 88 % over 23 h the

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contribution of GNG would have to be more than 100 % in the final period.

The reason why the NMR method may be misinterpreted as giving a constant rate of glycogenolysis is that the rate of hepatic glycogenolysis is calculated by finding the best fit of the measured glycogen concentrations to a line by the method of least squares. If the measured concentrations are set to a straight line the rate must be constant. However, what is sought is an overall rate; Shulman and co-workers (Rothman *et al*. 1991) did analyse segments with time. By the ^2H_2O method the contribution of GNG increases and hence the contribution of glycogenolysis decreases.

The contribution of GNG is then likely to be over-estimated in the diabetic subject by the NMR method (88 % throughout 23 h of fasting). The reason(s) is unclear, but may be due to the administration of as much as 150 g carbohydrate (of which 800 mg/g was glucose) in the meal. If at the time when glycogen concentration is first measured the glucose concentration is still higher than the basal level, or the extracellular space in which glucose is distributed is increased, the resulting glucose from the meal could be attributed to GNG. Also, carbohydrate unabsorbed at the time of the initial measurement of glycogen content would account for an overestimate of GNG, and in the diabetic subject decreased absorption due to gastroparesis could play a role, although this possibility is less likely with a liquid meal. Hourly absorption of only 1·5 g carbohydrate after the initial glycogen measurement by NMR would result in an overestimation of the contribution of GNG of approximately 15 %. This level of absorption would not be reflected in a change in blood glucose concentration, nor could it be detected by the appearance in blood of [^{2}H]glucose added to the meal (Petersen *et al*. 1996). Except in prolonged fasting, liver volume changes cannot be determined within the limits of the method, so volume has only been measured once or twice. However, changes in liver volume could generally have only a small effect on the estimates. Estimates of the contribution of GNG obtained by the ^{13}C NMR spectroscopy method are unaltered by glycogen cycling, pentose-cycle activity, transaldolase reactions, and triose phosphate cycling.

Discussion

Two other methods have recently been introduced to quantify GNG in human subjects. The first depends on the extent of enrichment of ^{13}C in C-3 and C-4 of blood glucose after $NaH^{13}CO_3$ administration. The extent of enrichment depends on the fixing of $^{13}CO_2$ by pyruvate to form oxaloacetate during GNG (Esenmo *et al*. 1992). However, because this enrichment is affected by the metabolism of oxaloacetate in the Krebs cycle, quantification requires estimation of the rate of GNG relative to Krebs-cycle flux (Magnusson *et al*. 1991; Esenmo *et al*. 1992). While reasonable estimates of GNG have been obtained (Landau *et al*. 1995*a*; Diraison *et al*. 1998, 1999), the method seems less attractive then the other methods because it is tedious to perform and relies on many assumptions.

The other method estimates the fractional contribution of GNG several days after beginning a diet in which the carbohydrate is relatively ^{13}C-enriched (Gay *et al*. 1994). Due to their relative quantity, ^{13}C enrichment in body protein and lipid should be essentially unchanged by the diet. On the other hand, because of the relatively small amount of hepatic glycogen in the body and its turnover, hepatic glycogen should be enriched with the ^{13}C in the diet. After several days on the diet, blood glucose during fasting should have an enrichment which is dependent on the contribution of glycogenolysis and the GNG substrates, specifically glycerol and amino acids. ^{13}C enrichment in glycogen which has undergone oxidation is calculated from breath $^{13}CO_2$ and gas exchange. Again, because of the size of the lipid and protein pools, amino acid and lipid oxidation are presumed to be a negligible source of the $^{13}CO_2$. Measurements are made at rest so that the oxidation of muscle glycogen is assumed to be insignificant. The enrichment in glycogen which has undergone oxidation is then assumed to be that of hepatic glycogen. In accordance with expectation, in the post-absorptive state the enrichment of glycogen which has undergone oxidation was found to be the same as that of dietary carbohydrate after 3 d on the diet. The enrichment in plasma glucose was half that of the glycogen, suggesting that 50 % of endogenous glucose released into the circulation was derived by GNG from glycerol and/or amino acids. Glucose formed by cycling between plasma glucose and C_3 compounds, i.e. Cori and glucose–alanine cycling, is not included in the estimate.

Of the four main methods which have been described, the first (heavy-water method) is probably the easiest to perform, since it requires the ingestion of ^2H_2O and sampling of blood. The ^{13}C NMR method requires measurements using instruments which are operational in only a few centres in the world. Instead of measuring GNG directly this method is based on the difference between the rates of glucose production and depletion of glycogen content. The ^{13}C NMR method provides measurement over an extended period rather than measurement at the time of sampling. Also, the time at which glucose production is measured relative to the time at which measurements of glycogen content are made can prove a problem, because the subject needs to be transported from the instrument to measure glucose production.

^{13}C-labelled GNG substrates and [U-$^{13}C_6$]glucose can be infused easily, but they are more expensive than ^2H_2O. Methods for the preparation of derivatives for measurements of isotopomers of glucose and lactate are established in many laboratories and are easier to perform than the formation of HMT from C-2 and C-5 positions of glucose. Learning the enzymic and chemical procedures for the isolation of the HMT may take weeks, but once learned can become routine for a laboratory.

A major limitation of the ^2H_2O method can be the inability to restudy a patient for several weeks. Evidence for essentially complete exchange of the H bound to C-2 of glucose, whether formed by GNG or glycogenolysis, allows the use of the ^{2}H enrichment at C-5:enrichment at C-2 for the estimation of GNG in a shorter interval after administration of ^2H_2O than is possible after administration of labelled glucogenic substrates in the MIDA method or [U-$^{13}C_6$]glucose in the [U-$^{13}C_6$]glucose isotopomer method. If there is doubt as to the completeness of the exchange, GNG can be estimated from the ^{2}H at

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C-5:^{2}H in body water in as short a time interval as with the other two methods.

In order to prepare an HMT containing C-5 with its H, 2 mg glucose (possibly less) is required. However, this amount of glucose may exceed the amount that can be collected in small children. The MIDA and [U-$^{13}C_6$]glucose isotopomer methods require less glucose. Only about 0·4 mg is needed to prepare an HMT for assay of the enrichment at C-6, and between 0·5 and 1·0 mg is required for the preparation of the HMT containing C-2 with its H. ^2H_2O has been used with pregnant women to measure GNG (Kalhan *et al*. 1997). However, despite the precautions taken, the small dose given and evidence relating to the safety of ^2H_2O, the administration of ^2H_2O to pregnant women, particularly during the first trimester cannot in general be recommended.

When glucose is infused into a subject, the rate of appearance of glucose in blood is the sum of the rates of the infusion and of glucose production via glycogenolysis and GNG. Applying the MIDA and [U-$^{13}C_6$]glucose methods yields the proportion of the rate of glucose appearance which is attributable to GNG. The fractional contribution of GNG to glucose production is slightly higher, since glucose production equals the rate of glucose appearance minus the rate of glucose infusion. The ^2H_2O method gives a direct measurement of the fractional contribution of GNG to glucose production. However, estimation of the contribution of GNG to glucose production then depends on estimating glucose production by subtracting the rate of glucose infusion from the rate of glucose appearance.

Unfortunately, the finding that the contribution of GNG is underestimated when using ^{13}C-labelled glycerol in the MIDA method has resulted in a focussed investigation into whether results with glycerol are valid. The results can be valid if sufficient glycerol is given, perhaps together with substrates that slow glycerol utilization along the liver lobule. However, the administration of glycerol at this level along with other labelled substrates (e.g. glucose to measure glucose production and galactose which is presumed to measure glycogen turnover) may perturb the fasting state. The underestimates obtained when [^{13}C]glycerol is administered are perhaps more important in emphasizing a major limitation in the general use of MIDA, i.e. the need to demonstrate or assume a single precursor enrichment. Quantification by the method of Tayek & Katz (1997) is dependent on the confidence that can be placed on their accounting for the several processes that can result in large underestimates.

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