Hepatic Glucose-6-Phosphatase Flux and Glucose Phosphorylation, Cycling, Irreversible Disposal, and Net Balance In Vivo in Rats. Measurement Using the Secreted Glucuronate Technique

Marc K. Hellerstein, Richard A. Neese, Amy Letscher, Peter Linfoot, and Scott Turner

Measurement of hepatic glucose production (HGP) by standard isotope dilution reveals only the net release of glucose from the liver, not the flux across glucose-6-phosphatase ([G6Pase] or total hepatic glucose output), hepatic glucose cycling (HGC), irreversible glucose disposal into glycogen in the liver (hepatic Rd), or net hepatic glucose balance. We describe two independent isotopic techniques for measuring these parameters in vivo, both of which use secreted glucuronate (GlcUA). HGC can be quantified by measuring a correction factor for glucose label retained in hepatic glucose-6-phosphate (G6P), sampled as GlcUA. A complementary technique for measuring total hepatic glucose output is also described (reverse dilution), requiring administration of no labeled glucose but instead a labeled gluconeogenic precursor and unlabeled glucose. Hepatic Rd is calculated by multiplying the rate of appearance (Ra) of hepatic UDP-glucose ([UDP-glc] based on dilution of labeled galactose in GlcUA) times the direct entry of glucose into hepatic UDP-glc and the fraction of labeled UDP-glc retained in the liver. The sum of hepatic Rd plus HGC represents the total hepatic glucose phosphorylation rate. Rats received intravenous (IV) glucose infusions at a rate of 15 to 30 mg/kg/min after a 24-hour fast. Despite a suppression of net HGP more than 50%, total hepatic glucose output was not significantly decreased, because of increased HGC. Total hepatic glucose output calculated by reverse dilution yielded similar results during IV glucose infusions at 15 mg/kg/min, although values were higher than obtained by the correction-factor method at 30 mg/kg/min. The fraction of labeled UDP-glc released into blood glucose, representing a hepatic glycogen cycle, decreased from 35% (fasted) to nearly 0% (IV glucose 30 mg/kg/min). Hepatic Rd was 1.4, 4.6, and 7.5 mg/kg/min (fasted and IV glucose 15 and 30 mg/kg/min, respectively); total hepatic glucose phosphorylation increased substantially (from 4.2 to 8.5 to 12.7 mg/kg/min) and net hepatic glucose balance changed from negative to positive during IV glucose. In conclusion, hepatic G6Pase flux, glucose phosphorylation, HGC, disposal of glucose into glycogen, and net glucose balance can be measured noninvasively in vivo under various metabolic conditions by techniques involving the GlcUA probe.

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THE LIVER is an organ of net glucose production in the postabsorptive state, but may contribute to net glucose disposal after a carbohydrate-containing meal. Moreover, hepatic uptake and phosphorylation of glucose may occur simultaneously as flux through hepatic glucose-6-phosphatase (G6Pase), representing hepatic glucose cycling (HGC) (Fig 1). The flux through G6Pase can be termed total hepatic glucose output, and is greater than the net release of glucose from the liver by the rate of hepatic phosphorylation of glucose.[1-4] After plasma glucose is phosphorylated by the liver, it undergoes one of two fates: dephosphorylation back to plasma glucose or disposal within the liver, mainly as glycogen (Fig 1).

However, isotope dilution techniques for measuring hepatic glucose production (HGP) do not measure total hepatic glucose output. Labeled glucose molecules taken up by the liver and released back into the circulation do not result in dilution of the circulating pool.[1,5,6] Thus, isotopically measured HGP (Fig 1, pathway 2) is less than the flux through G6Pase (total hepatic glucose output, pathway 4) by the rate of HGC (pathway 3). HGC is defined here as the flux rate in the opposite direction to the net flux. The inability of isotope dilution techniques to quantify HGC is an important limitation for hypotheses concerning the role of G6Pase in the regulation of hepatic carbohydrate metabolism by substrates and hormones.[2-4,7,8] Some studies[2] have suggested that insulin/glucose does not reduce hepatic G6Pase flux, for example, but instead causes output to be balanced by increased hepatic glucose uptake. HGC also results in hydrolysis of adenosine triphosphate, which may contribute to dietary carbohydrate–induced thermogenesis. This is another reason that measurement of total hepatic glucose output and HGC would be desirable.

HGC is not the only potentially confounding process in measuring hepatic carbohydrate balance. As just noted, the liver can also contribute to the rate of irreversible glucose disposal into liver glycogen ([hepatic Rd], pathway 7). Irreversible disposal into liver glycogen must be differentiated from HGC. Although both processes involve initial phosphorylation of glucose by the liver, the former contributes to the isotopically measured rate of systemic glucose disappearance (Rd glucose), does not reduce measured HGP, and represents glucose disposal that does not have to be performed by extrahepatic tissues, whereas the latter is not included in measured HGP or Rd glucose and does not reduce the burden on extrahepatic tissues. Another cycle of interest is glycogen synthesis concurrent with glycogenolysis,[9] which represents an intrahepatic process that may be important under certain conditions but is separate from the G6Pase cycle in terms of both its measurement and its physiologic implications. If labeled plasma glucose passes all the way into liver glycogen while unlabeled glycogen is being released concurrently into the plasma glucose pool (glycogen-glucose cycle), HGP by dilution will be increased but net glucose balance across the liver by arteriovenous difference will

From the Department of Nutritional Sciences, University of California, Berkeley; and the Division of Endocrinology and Medicine, Department of Medicine, San Francisco General Hospital, University of California, San Francisco, CA.

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Address reprint requests to Marc K. Hellerstein, MD, PhD, Department of Nutritional Sciences, 119 Morgan Hall, University of California, Berkeley, CA 94720-3104.

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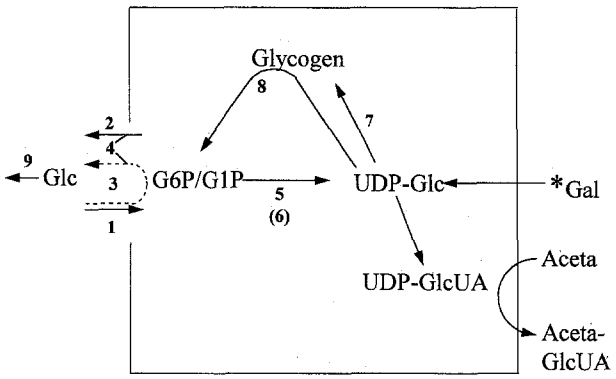

Fig 1. Model of hepatic glucose utilization pathways and cycles. Glc, glucose; Gal, galactose; Aceta, acetaminophen; G6P, glucose-6-phosphate; G1P, glucose-1-phosphate. Pathways: 1, hepatic glucose phosphorylation; 2, HGP (net); 3, HGC; 4, total hepatic glucose output (net HGP + HGC); 5, uncorrected Rd(H) (the contribution from pathway 7 to pathway 6, RaUDP-glc from all sources); 7, corrected Rd(H); 8, hepatic loss of labeled UDP-glc; 9, Rd(p).

not be increased. In contrast, labeled glycogen that is released to plasma glucose (pathway 8) will not be measured as HGP by dilution or by arteriovenous difference, representing another potential subtlety of the system.

There currently exist no isotopic techniques for directly measuring HGC, total hepatic glucose output, hepatic Rd, glycogen-glucose cycling, total hepatic phosphorylation of glucose, or hepatic glucose balance noninvasively in vivo. The most widely used technique for measuring various forms of HGC involves administration of doubly labeled glucose.[1] Loss of labeled hydrogen relative to labeled carbon signifies passage through particular enzymatic steps in the pathway of glycolysis/gluconeogenesis, depending on the carbon position to which the hydrogen is attached. However, these techniques are based on several assumptions that limit their utility. First, some metabolism of G6P must occur prior to release as free glucose for hydrogen extraction to occur (eg, passage through phosphoglucose isomerase to extract hydrogen from position C-2). Any retention of label in G6P will therefore result in underestimation of HGC. Second, glycolysis occurring in any tissue in the body followed by hepatic gluconeogenesis will decrease H/C ratios or cause label scrambling. Direct uptake and cycling of glucose by the liver alone cannot be inferred in vivo by this approach. Finally, distinctions among HGC (G6Pase cycle), irreversible glucose disposal by the liver (hepatic Rd), and glycogen cycles are not possible by this approach alone. Techniques for directly measuring parameters such as HGC, total hepatic glucose output, and hepatic Rd would therefore be useful, particularly for understanding the role of the liver in non–insulin-dependent diabetes mellitus.[2,10]

We describe here two experimental paradigms for measuring these parameters in vivo. One approach involves the use of two isotopic tracers (labeled glucose and galactose) with the glucuronate (GlcUA) probe.[11-14] In contrast to the double-labeled glucose approach based on measuring the loss of label in the hepatic G6P pool, this method for measuring HGC and total hepatic glucose output is based on measuring how much label has been retained in G6P. A second technique involves administering a labeled gluconeogenic precursor with the GlcUA probe and measuring dilution of plasma glucose relative to hepatic G6P. We present the theoretical basis of these methods and provide experimental data and calculations from studies in rats.

MATERIALS AND METHODS

Theory and Models

GlcUA correction factor for HGC and total hepatic glucose output. Standard dilution techniques for measuring HGP are based on the assumption that glucose entering the circulating pool is unlabeled.[1,5,6,15] Any input into the circulating glucose pool from the subpopulation of hepatic G6P molecules originating from plasma glucose (Fig 1, pathway 3) will have the same degree of labeling as the circulating pool and will not register as dilution. Therefore, no increase in plasma glucose flux will be measured. Plasma glucose that enters hepatic G6P and then is released back to the plasma represents HGC; accordingly, HGC is not measured by the standard dilution approach. However, HGC could be calculated if the isotopic labeling of hepatic G6P relative to circulating glucose (ie, the contribution from plasma glucose to hepatic G6P) were known during infusion of labeled glucose.[2,6,15] The standard dilution equation[5,6] is as follows:

$$\text{HGP (mg/kg/min)} = \frac{*\text{I}}{\text{plasma glucose labeling (SA or ME)}} - \text{I}, \quad (1A)$$

where *I is the infusion rate of labeled glucose (mg/kg/min or dpm/kg/min), I is the infusion rate of labeled plus unlabeled glucose (mass infusion rate), SA is the specific activity (dpm/mg), and ME is molar enrichment. The equations to correct for cycling are as follows:

$$\text{HGC (mg/kg/min)} = \text{HGP} \times \frac{\text{proportion ''labeled'' glucose released from G6P}}{\text{proportion ''unlabeled'' glucose released from G6P}} \quad (1B)$$

$$= \text{HGP} \times \frac{[\text{G6P/Glc}]}{1 - (\text{G6P/Glc})},$$

where G6P/Glc is the ratio of SA or ME of hepatic G6P to plasma glucose, "labeled" glucose represents glucose molecules with the same SA or ME as plasma glucose, and "unlabeled" glucose represents natural abundance glucose molecules.

Stated intuitively, equation 1B corrects for the subpopulation of hepatic G6P released into the circulation that fails to dilute plasma glucose because it came from and has the same isotopic content as the plasma glucose pool. As a numerical example, if 25% of hepatic G6P comes directly from plasma glucose, G6P will be 25% as isotopically enriched as plasma glucose during a constant infusion of [1-^{2}H$_1$]glucose, and HGC must represent $^{25}/_{75}$, or one third, of the rate of HGP measured by standard dilution. In kinetic terms, for every 3 mol noncycled glucose released, there is 1 mol cycled glucose released. This concept has been used previously by Rossetti et al,[2] Rognstad,[15] and our group[12] to estimate HGC.

If it is true that secreted GlcUA can be used to sample hepatic G6P labeling[14,16,17] (Fig 1), then equation 1B can be revised to

$$\text{HGC (mg/kg/min)} = \text{HGP} \times \frac{(\text{GlcUA/Glc})}{1 - (\text{GlcUA/Glc})}. \quad (1C)$$

Moreover, since total hepatic glucose output = HGP + HGC,

$$\text{total hepatic glucose output (mg/kg/min)} = \text{HGP} \times \left[1 + \frac{(\text{GlcUA/Glc})}{1 - (\text{GlcUA/Glc})}\right], \quad (1D)$$

where GlcUA/Glc is the ratio of SA or ME of secreted GlcUA to plasma glucose.

Rosetti et al[2] recently used an analogous strategy to calculate HGC, but they isolated UDP-glucose (UDP-glc) from rat liver and used it as an index of G6P labeling. We use GlcUA for this purpose based on the assumption that GlcUA comes from the same labeled pool of hepatic G6P that is used for secretion of plasma glucose, as demonstrated by us[18,19] and others.[14,16]

Irreversible disposal of plasma glucose into hepatic glycogen. Rd(H) represents steady-state plasma glucose disposal into liver glycogen. In isotopic terms, Rd(H) represents the portion of Rd glucose measured by standard isotope dilution that irreversibly leaves the circulation into liver glycogen. This is in contrast to HGC, which represents glucose that reenters the circulation from the liver. Since HGP exceeds hepatic glucose disposal under postabsorptive conditions, Rd(H) need not imply net glucose storage by the liver. The physiologic significance of Rd(H) in practical terms is that it represents systemic glucose disposal that does not have to be performed by peripheral tissues; the burden on peripheral tissues for glucose disposal is thereby reduced. In contrast, HGC does not reduce the burden on peripheral tissues for glucose disposal. Accordingly, one can subtract Rd(H) from Ra glucose to calculate "peripheral" tissue glucose disposal, Rd(p). One can also subtract HGP from hepatic Rd to calculate net hepatic glucose balance (net storage minus net release). It should be recognized that Rd(H) represents only plasma glucose disposal into liver glycogen and does not include other potential metabolic disposal routes of G6P in the liver, such as glycolysis or the hexose monophosphate shunt. These alternative pathways in the liver are included in Rd(p).

Rd(H) is measured by combining previously described techniques involving the GlcUA probe[9,10,17] with a new technique for measuring retention of labeled UDP-glc in the liver. Three measurements are required to calculate Rd(H), the fractional direct contribution from plasma glucose to hepatic UDP-glc, the Ra for hepatic UDP-glc (Ra UDP-glc), and the fractional retention of labeled UDP-glc in the liver:

fractional direct pathway contribution to UDP-glc

$$= \frac{\text{GlcUA labeling (SA or ME)}}{\text{plasma glucose labeling (SA or ME)}} \quad (2A)$$

during infusion of labeled glucose ([1-$^2H_1$]glc, [1-^{3}H]glc, or [U-$^{13}C_6$]glc). The method for measuring the direct pathway contribution to UDP-glc has been used extensively.[11-14,16,17]

Ra UDP-glc (mg/kg/min)

$$= \frac{*I}{\text{GlcUA labeling (SA or ME)}} - I(gal). \quad (2B)$$

*I is the infusion rate of [1-^{3}H]galactose or [1-^{2}H]galactose,[10,17,20] and I(gal) is the infusion rate of labeled plus unlabeled galactose (mass infusion rate). Because we use more than 99% enriched [1-$^2H_1$]galactose, I(gal) is essentially identical to *I.

Measurement of Ra UDP-glc by dilution of labeled galactose has been used previously by us[12,17,19] and by Rother and Schwenk.[16] The central assumption of this method is that galactose uptake is specific for the liver, so that the flux into hepatic UDP-glc can be calculated from the dilution of infused labeled galactose.[6,12]

To be rigorous, a correction has to be applied to subtract the return of labeled UDP-glc to the plasma glucose pool, since labeled glycogen that returns to plasma glucose does not result in dilution and does not increase steady-state HGP or Rd glucose, and thus does not represent irreversible glucose disposal in the liver but simply a roundabout form of HGC (pathway 8). This correction factor for fractional retention of labeled UDP-glc in the liver is directly calculated from the rate of label entry to plasma glucose relative to label entry to hepatic UDP-glc during infusion of [1-$^2H_1$]galactose (Fig 1). Fractional retention of labeled UDP-glc in the liver is calculated as

fractional retention of labeled UDP-glc

$$= 1 - (\text{recovery of infused galatose in plasma glucose}) \quad (2C)$$

$$= 1 - \left[\frac{[1\text{-}^2H_1]\text{glucose (ME)} \times \text{HGP}}{*I}\right],$$

where *I is the infusion rate of [1-$^2H_1$]galactose.

This measurement is based on the assumption of hepatic specificity of infused galactose[21-23]—ie, all [1-^{2}H]galactose passes through hepatic UDP-glc; and the assumption that the behavior of UDP-glc does not depend on its metabolic source—ie, UDP-glc from labeled galactose exhibits the same proportional retention in liver as UDP-glc from blood glucose. It is worth pointing out that no further assumptions concerning the precise fate of UDP-glc in the liver are required for this correction factor to be applicable—ie, whether all UDP-glc passes through hepatic glycogen (as we expect occurs) or there is reversal of the glucose-1-phosphate–uridyl transferase reaction (contrary to our expectation) (Fig 1), the recovery of labeled galactose in plasma glucose should reflect the recovery of labeled UDP-glc in blood glucose.

By combining equations 2A, 2B, and 2C, Rd(H) can be calculated:

Rd(H) (mg/kg/min)

$$= (\text{fractional direct pathway}) \times (\text{Ra UDP-glc}) \quad (2D)$$

$$\times (\text{fractional retention of labeled UDP-glc in the liver}).$$

By the difference between Rd(H) and total systemic glucose disposal (Rd glucose), Rd(p) can be calculated:

$$\text{Rd(p) (mg/kg/min)} = \text{Rd glucose} - \text{Rd(H)}. \quad (2E)$$

This calculation is based on the assumption that Rd glucose equals Ra glucose (ie, there is a metabolic steady state). As already noted, Rd(H) only accounts for hepatic storage of glycogen, not other hepatic disposal pathways.

Finally, hepatic glucose balance can be calculated as the difference between production and storage (assuming that systemic glucose Ra represents HGP):

$$\text{Hepatic glucose balance (mg/kg/min)} = \text{Rd(H)} - \text{HGP}. \quad (2F)$$

Total hepatic glucose phosphorylation. The sum of HGC plus Rd(H) represents the total hepatic glucose phosphorylation rate (Fig 1, pathway 1), again assuming that hepatic glycolysis and other nonglycogen pathways are quantitatively minor:

total hepatic glucose phosphorylation (mg/kg/min)

$$= \text{HGC} + \text{Rd(H)}. \quad (1E)$$

Reverse dilution technique for measuring total hepatic glucose output. An alternative technique for measuring total hepatic glucose output that does not involve infusion of labeled glucose will also be described. This technique uses the dilution principle but in a reverse manner than usual. Hepatic G6P can be taken as the precursor and plasma glucose the product when label enters hepatic G6P from within the liver—ie, when the direction of labeled carbon flow is outward from the liver into plasma glucose (Fig 2). When a labeled gluconeogenic precursor such as [2-$^{13}C_1$]glycerol is administered, for example, hepatic G6P will be more highly labeled, or at least as highly labeled, as plasma glucose. Accordingly, the ratio of isotopic enrichment or SA in plasma

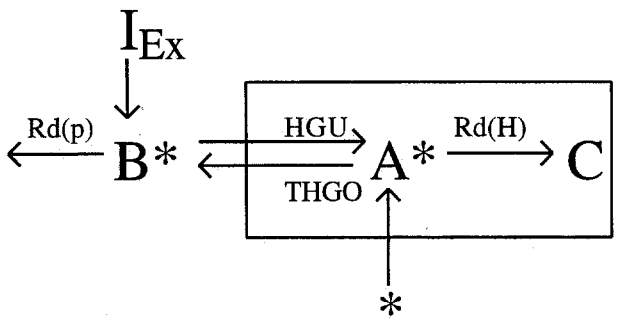

Fig 2. Reverse dilution model. B, plasma glucose; A, hepatic G6P; C, liver glycogen; I_{EX}, exogenous infusion of glucose; THGO, total hepatic glucose output; HGU, hepatic glucose uptake. Label enters plasma glucose through A, while disposal may be either peripheral [Rd(p)] or hepatic [Rd(H)].

glucose (the product) to hepatic G6P (the precursor) reflects the fraction of plasma glucose derived from hepatic G6P,

$$\text{plasma glucose from hepatic G6P (\%)} = \frac{\text{glucose enrichment (ME)}}{\text{G6P enrichment (ME)}} \times 100, \quad (3A)$$

when the isotope is known to have entered hepatic G6P from within the liver (eg, [2-^{13}C]glycerol or [2-^{13}C]acetate).

Under postabsorptive conditions, this ratio should be equal to (or very close to) unity, since plasma glucose is believed to derive essentially exclusively from hepatic G6P, absent renal gluconeogenesis. However, under conditions of exogenous glucose input such as IV glucose infusion, plasma glucose will have a lower isotopic enrichment than hepatic G6P. The degree to which plasma glucose is diluted relative to hepatic G6P reveals input from exogenous glucose (I_{Ex}) compared with input from the endogenous precursor (hepatic G6P). The latter input represents total hepatic glucose output (Fig 2). We call this method "reverse dilution" because the infused exogenous glucose is unlabeled and dilutes endogenous labeled glucose input, in contrast to the standard dilution technique in which infused exogenous glucose is labeled and is diluted by an unlabeled endogenous source. Since the cold glucose infusion rate (I_{Ex}) is known, total hepatic glucose output can be calculated as

$$\text{total hepatic glucose output (mg/kg/min)} = (I_{Ex}) \times \frac{\text{proportion of glucose from hepatic G6P}}{\text{proportion of glucose from exogenous source}} \quad (3B)$$

$$= (I_{Ex}) \times \frac{\text{Glc (ME)/G6P (ME)}}{1 - [\text{Glc (ME)/G6P (ME)}]},$$

where Glc (ME)/G6P (ME) is the ratio of plasma glucose to hepatic G6P isotope enrichment from [2-^{13}C]glycerol (Fig 2).

Assuming that GlcUA represents hepatic G6P, we can substitute:

$$\text{total hepatic glucose output (mg/kg/min)} = (I_{Ex}) \times \frac{\text{Glc (ME)/GlcUA (ME)}}{1 - [\text{Glc (ME)/GlcUA (ME)}]}. \quad (3C)$$

Again, the central assumption here is that GlcUA represents hepatic G6P under these conditions.[16,17,24]

A numerical example may be helpful. If the enrichment of plasma glucose is 50% of the enrichment of GlcUA during infusion of a ^{13}C-gluconeogenic precursor such as [2-$^{13}C_1$]glycerol plus cold glucose at 10 mg/kg/min, then the relative input into the plasma glucose pool from infused glucose (unlabeled) versus hepatic G6P (labeled) must be equal (ie, 10 mg/kg/min). Rognstad[15] has previously discussed this principle. A formal derivation of the calculation is provided in the Appendix.

Total hepatic glucose output calculated in this manner represents true flux across hepatic G6Pase—ie, it includes HGC (Fig 2). This is because the consequence of HGC is to reduce the isotopic enrichment of G6P and increase the isotopic enrichment of plasma glucose, thus altering the ratios in equations 3B and 3C and being registered as total hepatic glucose output.

Total hepatic glucose output calculated by reverse dilution has some unique and useful features. First, the assumptions of this approach are independent from those for the labeled glucose (GlcUA correction factor) method of measuring total hepatic glucose output (already described). Second, there is no need to infuse the labeled product (glucose) for reverse dilution—instead, one infuses unlabeled product. Moreover, problems related to exchange or slow equilibration of label in peripheral glucose pools[20,25] will have opposite effects on estimates of total hepatic glucose output by the correction factor versus reverse dilution techniques. With infusions of labeled glucose for standard isotope dilution, extrahepatic exchange of labeled glucose in and out of slow-turnover unlabeled pools decreases plasma glucose enrichment, thus increasing apparent HGP and total hepatic glucose output.[5,20,25] With reverse dilution, extrahepatic exchange of unlabeled for labeled glucose decreases plasma glucose enrichment, which reduces apparent total hepatic glucose output (equation 3C). Thus, if isotopic equilibration or exchange are concerns, one can calculate upper and lower bounds for total hepatic glucose output by applying standard dilution and reverse dilution techniques, respectively.

Experimental Methods

Isotopes and chemicals. [1-^{3}H]glucose, [1-^{3}H]galactose, and [2-^{14}C]acetate were purchased from ICN Radiochemicals (Irvine, CA). [1-$^2H_1$]galactose, [U-$^{13}C_6$]glucose, and [2-$^{13}C_1$]glycerol were purchased from Isotec (Miamisburg, OH). Sterile acetaminophen for IV administration was prepared by Dr L. Tomimatsu of the University of California–San Francisco School of Pharmacy, as described previously.[11,12]

Infusion studies. All infusions were performed on catheterized rats, as described in detail elsewhere.[9,24] Rats were allowed to recover for 48 hours after placement of intrajugular catheters for infusions. The infusions were performed for 4 to 9 hours. Acetaminophen was infused at 30 to 40 mg/kg/h. Radioisotopes ([1-^{3}H]glucose or [1-^{3}H]galactose) were infused at 10 to 15 μCi/h. [1-$^2H_1$]galactose was infused at 1 mg/kg/min. [2-$^{13}C_1$]glycerol was infused at 1 mg/kg/min.[24] IV glucose was infused at 15 mg/kg/min. Animals were fasted for 24 hours before starting infusions.

Isolation of metabolites and preparation for mass spectrometric analyses. Two metabolites must be isolated and sampled to apply these techniques: plasma glucose and secreted GlcUA. Glucose was isolated from deproteinized plasma by ion-exchange chromatography.[11,12] SA was determined, after lyophilization to remove ^3H_2O, by liquid scintillation counting (LS600IC; Beckman, Palo Alto, CA), with enzymatic measurement of glucose content as described previously.[11] SA is expressed as dpm per micromole, to allow comparison between glucose and GlcUA. For analyses of [^{2}H]glucose enrichment, the glucose pentacetate derivative was synthesized from glucose with acetic anhydride in pyridine.[22] For measurements of [^{13}C]glucose labeling, dimethyltetracetyl saccharic acid was synthesized and analyzed.[24,26,27]

Acetaminophen GlcUA was isolated from urine as described elsewhere.[11,12] Dimethyltriacetyl saccharic acid and methyltetracetyl GlcUA derivatives were synthesized for gas chromatographic/mass spectrometric (GC/MS) analyses.[17,18,27]

GC/MS analyses. Glucose pentacetate and methyltetracetyl GlcUA were analyzed by GC/MS under chemical ionization conditions.

Selected ion monitoring (SIM) was used with chemical ionization (methane). For glucose pentacetate, GC/MS conditions were as described previously.[24] For the dimethyltriacetyl saccharic acid derivative of glucose or GlcUA,[17,27] SIM of *m/z* 347-349 was performed with a DB-17 column (60 m).

The methyltetracetyl GlcUA analysis was also made with a 60-m DB-17 column and SIM of *m/z* 317-319. The GC/MS instrument was Hewlett-Packard model 5971 (Hewlett-Packard, Palo Alto, CA).

RESULTS

Total Hepatic Glucose Output and HGC

Results are shown for rats infused with acetaminophen, [1-^{3}H]glucose, and [2-$^{13}C_1$]glycerol to calculate total hepatic glucose output by both the labeled glucose/GlcUA correction factor and reverse dilution techniques (Table 1). IV glucose was infused at 15 kg/mg/min over the final 5 hours of the 9-hour isotope infusion, after the first 4-hour infusion in the fasted state. Sequential arterial blood samples and urine collections were taken. Isotopic steady state was attained with fasting, as we have shown previously,[11,17] and during IV glucose (Fig 3) for the isotope dilution and MIDA measurements. The data in Table 1 include some animals for which Ra glucose and fractional gluconeogenesis data have been presented previously.[17] In the fasted state, HGP by standard dilution of [1-^{3}H]glucose was 6.0 ± 0.7 mg/kg/min, and the [^{3}H]GlcUA/[^{3}H]glucose ratio was 0.31 (Table 1). By equation 1D, HGC was therefore (6.0) × (0.31/0.69) = 2.7 mg/kg/min and total hepatic glucose output was 6.0 + 2.7 = 8.7 mg/kg/min. From [2-^{13}C]glycerol, the ratio of [^{13}C]glucose/[^{13}C]GlcUA was 1.03 under fasting conditions (not significantly different from unity), as we reported elsewhere.[24,28] Reverse dilution thereby confirmed the absence of input from sources other than hepatic G6P into plasma glucose. During IV glucose infusion at 15 mg/kg/min, Ra glucose was 19.2 ± 1.6 mg/kg/min and HGP was 4.2 ± 1.6 mg/kg/min. The correction factor increased to 0.48 (ie, 48% of hepatic G6P was derived from labeled plasma glucose), so total hepatic glucose output was calculated to be 8.1 and HGC 3.9 mg/kg/min. The reverse dilution calculation yielded similar results. The ^{13}C-glucose/^{13}C-GlcUA ratio was 0.39 during IV glucose infusion at 15 mg/kg/min, so total hepatic glucose output was 15 mg/kg/min × (0.39/0.61) = 9.6 mg/kg/min and HGC was 9.6 − 4.2 = 5.4 mg/kg/min.

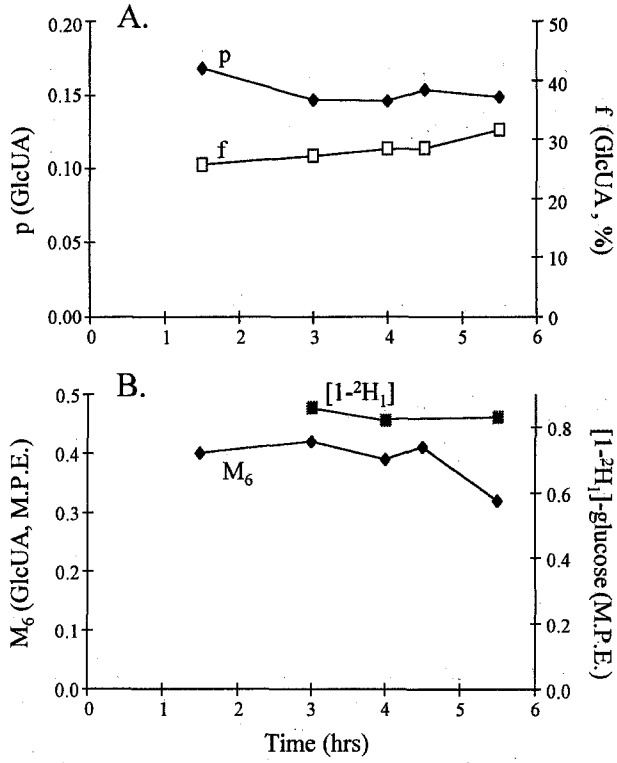

Fig 3. Isotopic steady state attained in urinary GlcUA and blood glucose during infusion of IV glucose with [2-$^{13}C_1$]glycerol, [U-$^{13}C_6$]glucose, and [1-$^{2}H_1$]galactose. (A) Gluconeogenic precursor pool enrichment (p) and fraction of glucose from gluconeogenesis (f) values in GlcUA. (B) M_6-GlcUA and [1-$^{2}H_1$]glucose. Steady states during fasting have been shown by us previously,[17,29] and a steady state of [1-$^{2}H_1$]galactose in GlcUA during IV glucose was also reported by us previously.[29]

Table 1. Total Hepatic Glucose Output and HGC by Labeled Glucose/GlcUA (correction factor) and Reverse Dilution Techniques in Rats

Experimental Conditions and Isotopes	Primary Measurements						Derived Measurements			
	Ra Glucose (mg/kg/min)	HGP (mg/kg/min)	f		Reverse Dilution Ratio	Correction Factor ([^{3}H]GlcUA/[^{3}H]Glucose)	Correction Factor (mg/kg/min)		Reverse Dilution (mg/kg/min)	
			Glucose	GlcUA			HGC	THGO	HGC	THGO
Fasted, [1-^{3}H]glucose + [2-$^{13}C_1$]glycerol (n = 10)	6.0 ± 0.7	6.0 ± 0.7	0.90 ± 0.02	0.88 ± 0.03	1.03 ± 0.04	0.31 ± 0.02	2.7 ± 0.4	8.7 ± 0.7	—	—
IV glucose 15 mg/kg/min [1-^{3}H]glucose + [2-$^{13}C_1$]glycerol (n = 6)	19.2 ± 1.6	4.2 ± 1.6	0.18 ± 0.01	0.46 ± 0.04	0.39 ± 0.02	0.48 ± 0.03	3.9 ± 0.4	8.1 ± 0.6	5.4 ± 0.9	9.6 ± 0.6
[1-^{3}H]glucose + [2-$^{13}C_1$]acetate (n = 12)	19.5 ± 1.2	4.5 ± 1.2	0.64 ± 0.10	1.79 ± 0.30	0.36 ± 0.03	0.46 ± 0.02	3.8 ± 0.5	8.3 ± 0.8	3.9 ± 0.7	8.4 ± 0.8
IV glucose 30 mg/kg/min + [2-$^{13}C_1$]glycerol (n = 6)	33.2 ± 1.8	3.2 ± 1.8	0.08 ± 0.02	0.30 ± 0.07	0.27 ± 0.02	0.62 ± 0.04	5.2 ± 0.4	8.4 ± 0.8	7.9 ± 1.1	11.1 ± 1.2

NOTE. Data are the mean ± SE. HGC (correction factor method) = correction factor/1 − correction factor × HGP. THGO (reverse dilution [RD] method) = (RD Ratio/1 − RD Ratio) × (I_{Ex}).

Abbreviation: THGO, total hepatic glucose output; f, traditional contribution from gluconeogenesis (for [2-$^{13}C_1$] acetate, relative enrichments are shown instead of f).

In principle, any carbon-labeled substrate that labels G6P from within the liver can be used for reverse dilution calculations. Infusion of sodium [2-^{13}C]acetate was therefore compared in a separate set of experiments under otherwise identical conditions (with [^{3}H]glucose, acetaminophen, and IV glucose). By reverse dilution, the ^{13}C-glucose/^{13}C-GlcUA ratio was 0.36, resulting in total hepatic glucose output = 15 mg/kg/min × (0.36/0.64) = 8.4 mg/kg/min and HGC = 8.4 − 4.5 = 3.9 mg/kg/min. By the correction factor method, [^{3}H]glucose/[^{3}H]GlcUA = 0.46, HGC = (4.5) × (0.46/0.54) = 3.8 mg/kg/min, and total hepatic glucose output = (4.5 + 3.8) = 8.3 mg/kg/min. The two estimates were therefore very close under conditions of IV glucose infusion at 15 mg/kg/min.

At higher IV glucose infusion rates (30 mg/kg/min), reverse dilution and correction factor results no longer correlated so well (Table 1). By the correction factor technique, total hepatic glucose output (8.4 mg/kg/min) was unchanged from fasting or IV glucose at 15 mg/kg/min, but HGC (5.2 mg/kg/min) was significantly increased. However, by reverse dilution, total hepatic glucose output (11.1 mg/kg/min) and HGC (7.9 mg/kg/min) were even higher. We conclude that the reverse dilution technique is less reliable than the correction factor technique at high glucose infusion rates (discussed later). However, neither method indicated that total hepatic glucose output was suppressed by IV glucose administration.

Rd(H)

Ra UDP-glc was 4.5 ± 0.2 mg/kg/min. Multiplying by the direct pathway contribution to UDP-glc flux (0.32 ± 0.3) yields the absolute rate of direct plasma glucose entry into hepatic UDP-glc (1.4 ± 0.2 mg/kg/min; Table 2). This value represents uncorrected hepatic Rd.[10,17] However, it is necessary to correct this value for labeled UDP-glc that is retained in the liver, because labeled UDP-glc that passes to plasma glucose (presumably through liver glycogen, pathway 8 and then 3; Fig 1) does not represent irreversible hepatic glucose disposal, but HGC instead. During fasting, retention of [1-$^{2}H_1$]galactose by the liver (Table 2) was 65.3% ± 7.3% (range, 38.3% to 81.4%). During IV glucose 30 mg/kg/min, fractional retention of [1-$^{2}H_1$]galactose was almost 100% (99.2% ± 0.3%) in all animals. Direct glucose retained by the liver, or corrected hepatic Rd (uncorrected hepatic Rd × hepatic retention of labeled [1-$^{2}H_1$]galactose), was therefore 7.4 ± 1.1 mg/kg/min during IV glucose 30 mg/kg/min, significantly increased from 0.9 ± 0.2 mg/kg/min in the fasted state (Table 2). Total hepatic phosphorylation of glucose (HGC plus corrected hepatic Rd) also increased significantly during IV glucose infusion, from 3.6 ± 0.3 to 12.6 ± 1.3 mg/kg/min. Corrected Rd(p) (Ra glucose corrected hepatic Rd) was 5.1 ± 0.8 mg/kg/min (fasted) and 25.8 ± 2.4 mg/kg/min (IV glucose 30 mg/kg/min). Irreversible hepatic Rd therefore accounted for about 25% (7.4 of 30 mg/kg/min) of infused glucose. Net hepatic glucose balance went from negative (−5.1 ± 0.8 mg/kg/min) under fasting conditions to positive (+4.2 ± 1.7 mg/kg/min) during IV glucose 30 mg/kg/min.

Model of Fluxes

A schematic model of hepatic carbohydrate fluxes under conditions of fasting and IV glucose 30 mg/kg/min is shown in Fig 4.

DISCUSSION

The two techniques presented here for measuring total hepatic glucose output and HGC are based on independent assumptions and are therefore complementary. The one shared assumption for both techniques is that secreted GlcUA accurately represents labeling of the hepatic G6P pool from which plasma glucose is formed. Our group and others[14,16,24,28,29] have presented evidence elsewhere that GlcUA indeed represents the G6P pool for glucose secretion and glycogen synthesis from labeled glucose or gluconeogenic precursors.

The reverse dilution technique represents a novel alternative to standard isotope dilution for measuring the flux of a metabolite such as plasma glucose. As noted earlier, the assumptions and direction of the error are different for reverse dilution and standard dilution, making them complementary and potentially useful for cross-validation. Limitations of the reverse dilution technique should also be pointed out. First, this approach requires concurrent infusion of unlabeled glucose. The calculation is not possible in the absence of cold glucose infusion. Reverse dilution therefore cannot be used under true fasting conditions (Table 1). Another limitation is that any experimental error is magnified as the glucose infusion rate increases. This is because the glucose/GlcUA labeling ratio is multiplied by the glucose infusion rate (equation 3C) and also because incorporation of gluconeogenic label into plasma glucose tends to be low at high glucose infusion rates (Table 1), so a larger analytic coefficient of variation is introduced. At high glucose infusion rates, small variations in the glucose/GlcUA ratio will therefore have a relatively large effect on calculated total hepatic glucose output. Consistent with these considerations, results from reverse dilution no longer correlated well with the correction factor method at IV glucose 30 mg/kg/min (Table 1). The GlcUA correction factor technique is, in our

Table 2. Hepatic Glucose Disposal

Dietary Condition	Uncorrected Rd(H) (mg/kg/min)	Hepatic Retention of Labeled UDP-glc (%)	Corrected Rd(H) (mg/kg/min)	Total Hepatic Glucose Phosphorylation (mg/kg/min)	Rd(p) (mg/kg/min)	Hepatic Glucose Balance (mg/kg/min)
Fasted	1.4 ± 0.2	65.3 ± 7.3	0.9 ± 0.2	3.6 ± 0.3	5.1 ± 0.8	−5.1 ± 0.8
IV glucose 15 mg/kg/min	4.6 ± 0.9	ND	ND	ND	ND	ND
IV glucose 30 mg/kg/min	7.5 ± 1.1	99.2 ± 0.3	7.4 ± 1.1	12.6 ± 1.3	25.8 ± 2.4	+4.2 ± 1.7

NOTE. [1-^{3}H]glucose and [1-^{2}H]galactose were infused IV in rats. Ra UDP-glc and direct pathway data have been presented elsewhere.[17] Data for HGC (from which total hepatic glucose phosphorylation was calculated) were obtained by the correction factor method in Table 1. Uncorrected Rd(H) = Ra UDP-glc × fractional direct pathway contribution; corrected Rd(H) = uncorrected Rd(H) × hepatic retention of labeled UDP-glc; total hepatic glucose phosphorylation = HGC + corrected Rd(H); extrahepatic glucose disposal = Rd glucose − corrected Rd(H); hepatic glucose balance = corrected Rd(H) − HGP, where negative represents release and positive represents uptake of glucose.

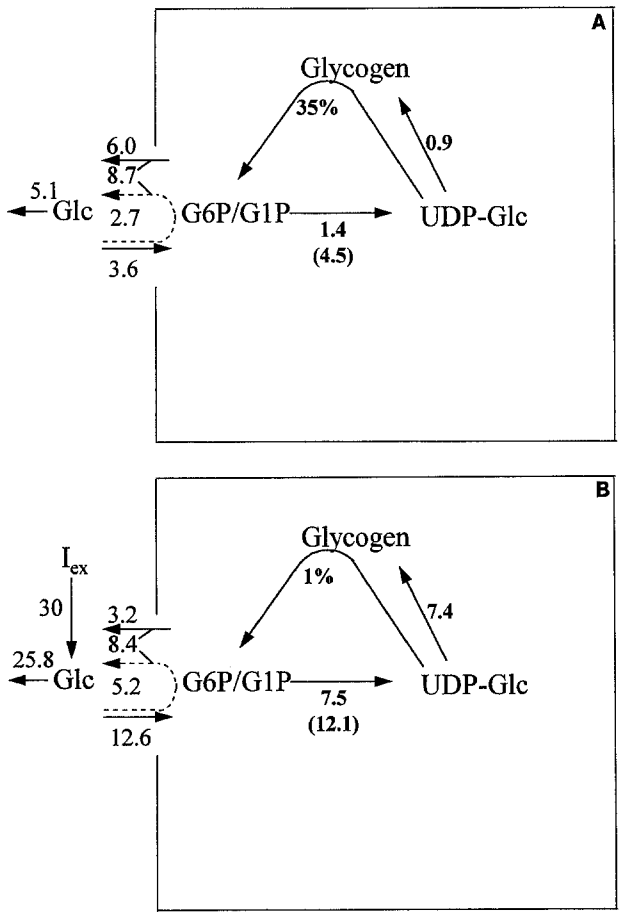

Fig 4. Hepatic glucose balance and utilization during fasting (A) and IV glucose 30 mg/kg/min (B) in rats. Pathways are as identified in Fig 1. Numbers represent the flux (mg/kg/min) through each pathway.

opinion, more reliable under these conditions. In contrast, estimates of total hepatic glucose output and HGC were similar by the two techniques during the lower rate of glucose infusion (15 mg/kg/min; Table 1), when incorporation of gluconeogenic label into plasma glucose was not as suppressed.

The methods presented here are based on certain metabolic assumptions that are worth discussing in more detail. The calculation of Ra UDP-glc—and from this, disposal of plasma glucose into hepatic glycogen [Rd(H)] and total glucose phosphorylation by the liver—assumes hepatic specificity of administered galactose. Available biochemical and physiological[21-23] evidence supports this assumption in a general way, but it is difficult to prove that 100% of labeled galactose is in fact taken up by the liver. If a substantial fraction of galactose is taken up by extrahepatic tissues (eg, >15% to 20%), Ra UDP-glc will be proportionately over estimated. Also, we assume that alternative disposal routes of hepatic G6P, such as glycolysis or the hexose monophosphate shunt, are quantitatively minor.[18] It should be noted that this assumption is not critical to the measurements described here, because Rd(H) refers only to disposal of glucose into liver glycogen. Other metabolic fates of G6P in the liver are lumped with "peripheral" glucose disposal.

An important assumption of the method is that a metabolic and isotopic steady state is present. In particular, the calculations of isotope dilution (Ra glucose and Ra UDP-glc) are based on the steady-state assumption.[5,6,15,20,25] The fractional contribution calculations (direct pathway contribution from plasma glucose to UDP-glc, GlcUA correction factor for hepatic glucose cycling, and recovery of labeled UDP-glc in plasma glucose) do not require a metabolic steady state: as for any precursor-product relationship,[5,30] the measured contribution accurately represents the fractional contribution during the period of isotope administration regardless of whether the contribution changes over time. In these animals[17,29] (Fig 3), isotopic and metabolic steady states were documented for isotope dilution measures (Ra glucose and Ra UDP-glc), as well as MIDA calculations (fractional contribution from gluconeogenesis to glucose and GlcUA).

Both methods indicate that the flux across hepatic G6Pase is not reduced by administration of IV glucose. As the glucose infusion rate increased, HGC represented an increasing proportion of total hepatic glucose output, so that net glucose release (HGP) decreased. This explanation for reduced HGP is consistent with previous results.[2,17] These results also confirm that the liver changes from negative to positive glucose balance when IV glucose is administered after a 24-hour fast (Table 2). It should be noted that positive hepatic glucose balance need not be quantitatively identical to glycogen synthesis during glucose infusion, if the indirect pathway of glycogen synthesis is operating.[7,11-13]

Our finding that G6Pase flux remains active during administration of exogenous glucose supports some previously reported experimental results.[31] Others[32] have concluded that glucose plus insulin stops G6Pase flux almost completely. However, the report of Moore et al[32] was based on insensitive techniques (requiring measurement of small differences between ^{3}H-glucose SAs across the liver to identify flux through G6Pase). This is an important question in hepatic metabolism, and we believe that the results presented here and previously[12,17] provide strong evidence that flux through hepatic G6Pase remains active during glucose infusions.

Irreversible hepatic glucose disposal, ie, corrected Rd(H), has very different metabolic implications compared with the simple glucose phosphorylation/dephosphorylation cycle. The effect of exogenous glucose on hepatic Rd may be relevant to hyperglycemic states such as non–insulin-dependent diabetes,[2,10] since hyperglycemia clearly increases the direct pathway fractional contribution to hepatic UDP-glc.[11,13] One possibility is that hepatic Rd serves as a spillover pathway for glucose flux that cannot be taken up by peripheral tissues. This question should be directly answerable using the techniques described here.

The retention of labeled UDP-glc in liver glycogen was estimated by the proportion of [1-^{2}H$_1$]galactose that appeared in plasma as glucose (Table 2). The results are of physiologic and methodologic interest. Under fasting conditions, about one third (34.7%) of newly synthesized glycogen was released back to glucose over the course of a 4-hour isotope infusion study. In humans, the value for this same parameter after an overnight fast was about 20%, and during a 60-hour fast, about 35%.[26] Thus, a last-in/first-out model of glycogen turnover does not strictly apply to glycogen mobilization during a fast, but significant glycogen-glucose cycling of label does occur.

In summary, an experimental approach and model is presented here for measuring the cycling, total output, phosphorylation, disposal, and balance of glucose noninvasively in vivo in a relatively straightforward manner. Infusion of two or three isotopic tracers is required (labeled glucose and galactose, plus a gluconeogenic precursor if reverse dilution is used) along with acetaminophen, but only two metabolites must be analyzed (plasma glucose and secreted GlcUA). Application of both techniques described here during IV glucose infusion indicates that the flux through hepatic G6Pase is not suppressed but total glucose phosphorylation, HGC, and glucose disposal into liver glycogen are stimulated by administration of exogenous glucose. It will be of interest to correlate these noninvasive estimates with directly measured balances across the liver.[33]

APPENDIX

An isotope and chemical balance approach can be used for formal derivation of the reverse dilution equations. At isotopic steady state, input of label into the product B ($*_{IN}$) must equal exit of label from B ($*_{OUT}$; Fig 2),

$$*_{IN} = *_{OUT},$$

$$*_{IN} = \text{THGO} \times \text{A}^*, \text{ and}$$

$$*_{OUT} = (\text{Rd(p)} \cdot (\text{B}^*) + (\text{HGU} \cdot \text{B}^*),$$

where B* = SA or ME of label in B, A* = SA or ME of label in A, HGU = hepatic glucose uptake, and Rd(p) = rate of peripheral glucose disposal. Accordingly,

$$\text{THGO} \times \text{A}^* = (\text{Rd(p)} \cdot \text{B}^*) + (\text{HGU} \times \text{B}^*). \quad (1A)$$

$$\text{Rearranging, THGO} = \text{B}^*/\text{A}^* [\text{Rd(p)} + \text{HGU}]. \quad (1B)$$

Chemical flux into B also must equal chemical flux out of B, $\text{Flux}_{IN} = \text{Flux}_{OUT}$, and

$$I_{Ex} + \text{THGO} = \text{Rd(p)} + \text{HGU}, \quad (2)$$

where I_{Ex} = cold glucose infusion rate.

We can then substitute for [Rd(p) + HGU] from Eq 2 into Eq 1B, to reduce the equation to measurable parameters and solve for total hepatic glucose output (THGO):

$$\text{THGO} = \text{B}^*/\text{A}^*(I_{Ex} + \text{THGO})$$

$$\text{THGO} - \text{THGO}(\text{B}^*/\text{A}^*) = I_{Ex}(\text{B}^*/\text{A}^*) \quad (3)$$

$$\text{THGO}\,(1 - \text{B}^*/\text{A}^*) = I_{Ex} \cdot \text{B}^*/\text{A}^*,$$

$$\text{and therefore, THGO} = I_{Ex} \cdot \frac{\text{B}^*/\text{A}^*}{1 - \text{B}^*/\text{A}^*}. \quad (4)$$

It is important to note that the flux that cycles through hepatic glucose uptake and total hepatic glucose output (ie, the glucose phosphorylation/dephosphorylation cycle, HGC) does not affect the validity of the method. As HGC increases, the ratio B*/A* becomes closer to unity, which will increase the total hepatic glucose output calculation (Eq 4) in proportion to the rate of HGC, as it should.

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