The purposes of this study were to quantify the impact of the duration of infusion and choice of stable isotope of glucose on measures of glucose rate of appearance (glucose $R_a$) and to determine whether the differences observed were due to tracer recycling via the glycogen pool (direct pathway) or gluconeogenesis (indirect pathway). Six healthy adult volunteers were studied on four occasions in the postabsorptive state during infusions of [1-13C]glucose and [6,6-2H2]glucose: 2.5-h infusion of both (A), and 2.5-h infusion of one (B) and 14.5-h infusion of the other isotope (C), and 5-h infusion of [6,6-2H2]glucose and 2.5-h infusion of [1-13C]glucose (D). Infusion of both isotopes for 2.5 h resulted in similar glucose $R_a$ values. When compared with a 14.5-h infusion, the 2.5-h glucose tracer infusion overestimated glucose $R_a$ by 26–35%. Glucose 13C recycled via the Cori cycle, resulting in slower decay from the plasma pool and longer half-life of [1-13C]glucose compared with [6,6-2H2]glucose. There was no detectable release of [13C]glucose or [2H2]glucose tracer into the plasma pool after administration of glucagon. These data demonstrate that glucose $R_a$ varies not as a result of isotope cycling but as a result of differences in duration of isotope infusion regardless of the isotope used. This is most likely due to incomplete isotope and substrate equilibration with the 2.5-h infusion. The potential error was reduced by nearly 80% using a 5-h infusion of [6,6-2H2]glucose. These studies demonstrate that the duration of isotope infusion has significantly greater impact on quantitation of glucose $R_a$ than does the selection of isotope. Diabetes 51:3170–3175, 2002

We have previously demonstrated that during prolonged infusion, labeled leucine is presumably incorporated into protein and released into the vascular space, leading to an underestimation of leucine flux (1). Both carbon- and hydrogen-labeled glucose can potentially be recycled as the result of direct uptake of labeled glucose into and release from glycogen. Only carbon-labeled glucose, however, can potentially recycle via gluconeogenesis from labeled pools of lactate and pyruvate (2). Independent of whether tracer recycling occurs, duration of an isotope infusion may also affect the calculated glucose rate of appearance ($R_a$) if the tracer and tracee are not in nearly complete equilibrium. In normal subjects, the estimate of hepatic glucose production using a 2-h primed constant rate infusion of [6,6-2H2]glucose was significantly higher than that obtained after a primed 4-h isotope infusion (3). However, it was not clear from these studies whether this reduction was due to a difference in steady state or to a real physiological reduction in glucose $R_a$ over time. Kalhan et al. (2) measured glucose production in newborn infants who received a simultaneous infusion of [1-13C]glucose and [6,6-2H2]glucose during a 2-h period. The glucose production rate measured from the dilution of [1-13C]glucose was slightly but significantly lower than that measured with the [6,6-2H2]glucose. The investigators concluded that this difference was due to recycling of the glucose C-1 through the Cori cycle.

Use of isotopes of glucose to estimate the $R_a$ and/or rate of production of glucose in humans is a fundamental research tool for the clinical investigator involved in studies of glucose homeostasis. Despite wide application of the isotope dilution techniques, the factors that affect the findings of such studies are incompletely understood. The purposes of the present study were to quantify the impact of the duration of infusion and choice of stable isotope tracer on tracer recycling. Using a dual isotope infusion approach, we assessed how these measures are affected by 1) potential recycling of labeled glucose molecules via gluconeogenesis and/or glycogenolysis and 2) the equilibration of the tracer with the tracee.

RESEARCH DESIGN AND METHODS

Subjects. The protocol was approved by the Institutional Review Board for Human Subject Research at Baylor College of Medicine in Houston, TX. Informed written consent was obtained from six healthy, male, nonobese adult volunteers: 28 ± 2 years, BMI 23.7 ± 1.2 kg/m2 (mean ± SE). The subjects had a normal physical examination and screening laboratory studies (complete blood count, electrolytes, renal and liver function tests), were taking no medications, and had no family history of diabetes. Each subject was studied on four occasions separated by 1–2 months. The order of studies A, B, and C was randomized. Study protocol D was carried out in all six subjects once the results of A, B, and C were available on the first three subjects.

Experimental design. See Fig. 1 for graphic representation of protocols. Study protocol A. The subjects were instructed to consume a normal diet with a caloric distribution of 50% carbohydrate, 15% protein, and 35% fat during the week preceding the study. They were admitted to the Metabolic Research Unit at the Children's Nutrition Research Center in the afternoon and offered a standardized meal between 1730 and 1800 h. They were subsequently placed at bed rest and were fasted, with the exception of ad libitum water, until completion of study the following afternoon. Two intravenous catheters were introduced in each antecubital space under EMLA cream analgesia, one for isotope infusion and the other for blood sampling. A baseline blood sample was obtained before the initiation of isotope infusion. At 0800 h, [6,6-2H2]glucose and [1-13C]glucose were given as primed...
ensuring no interference from the [1-13C]glucose in the determination of the spectrometry (GCMS) using a quadrupole instrument. The electron impact prolonged infusion of [1-13C]glucose. The isotopic enrichments of the derivative of glucose was prepared as described previously (4,5). The isotopic commercial radioimmunoassay (Linco, St. Charles, MO). The penta-acetate and kept at 4°C until used. Separate solutions were prepared for each of the isotope infusions were discontinued and the catheter was immediately removed to guarantee the absence of any additional isotope being derived from the infusion catheter line. Samples were obtained at 15-min intervals during the next 3 h. At 3 h after discontinuing the isotopes, each subject received an intravenous bolus of 1 mg of glucagon and blood sampling continued at 10-min intervals for an additional 60 min. **Study protocol B.** Protocol B was identical to protocol A with the exception that the infusion of [1-13C]glucose was initiated at 2000 h on the day of admission (duration of infusion 14.5 h). **Study protocol C.** Protocol C was identical to protocol A with the exception that the infusion of [6,6-2H2]glucose was initiated at 2000 h on the day of admission (duration of infusion 14.5 h). **Study protocol D.** Protocol D was identical to protocol A with the exception that the infusion of [6,6-2H2]glucose was initiated at 0530 h (duration of infusion 5 h). **Isotopes.** [6,6-2H2]glucose (99% 2H) and [1-13C]glucose (99% 13C) were obtained from Cambridge Isotope Laboratories (Andover, MA). The isotopes were dissolved in 0.45% saline, and the solution was filtered through a 0.2-μm Millipore filter into sterile syringes. All sterile isotopes were prepared <48 h before study and maintained at 4°C until used. Separate solutions were prepared for each of the two tracers. **Analyses.** Blood samples were placed on ice, and the plasma was separated and kept at −70°C until assayed. Plasma glucose concentrations were determined by a glucose oxidase method (YSI glucose analyzer; YSI Yellow Springs, OH), and plasma glucagon concentrations were determined by a commercial radioimmunoassay (Linco, St. Charles, MO). The penta-acetate derivative of glucose was prepared as described previously (4,5). The isotopic enrichments of [6,6-2H2]glucose were measured by gas chromatography–combustion-isotope ratio mass spectrometry (Europa Scientific, ANCA-NT 2020, GC: HP 5890 with an HP 1701 column 30 m × 0.25 mm × 1 μm; Agilent Technologies, Wilmington, DE). Finally, the acetyl-pentafluorobenzyl derivative of lactate was prepared as described previously (6,7). The 13C enrichments in lactate were analyzed by negative chemical ionization GCMS as previously described (5,7). **Calculations.** Plasma glucose R0 at steady state was calculated using established equations for isotope dilution (8,9):

\[ R_0 = \left( \frac{E_t}{E_0} - 1 \right) \times 1 \]  
(1)

\[ E_t = E_0 e^{-kt} \]  
(2)

\[ t_{1/2} = \frac{\ln(2)}{k} = \frac{0.693}{k} \]  
(3)

The absolute amount of new tracer released from glycogen into the plasma pool (mmol/l) before and after administration of glucagon was calculated by multiplying the enrichment of the tracer (after subtracting the enrichment value obtained immediately before the glucagon bolus) by the glucose concentration.

**Statistics.** All values were expressed as mean ± SE. ANOVA procedure for repeated measures (using Fisher’s least squares difference for multiple comparisons) was used to test for differences in the mean glucose R0 glucose concentration, and the rate constant of isotope decay curve and t1/2 values in the various subgroups under study. Differences in the lactate enrichment at the end of 14.5- vs. 2.5-h infusion of [1-13C]glucose were tested using paired Student’s t test. P ≤ 0.05 was considered to indicate statistical significance. All statistical analyses were performed on a personal computer with the statistical program SPSS (version 8.0) for Windows.

**RESULTS**

**Plasma glucose concentrations.** Plasma glucose concentrations at steady state were similar on all four study occasions (5.59 ± 0.03, 5.34 ± 0.02, 5.50 ± 0.01, and 5.37 ± 0.01 mmol/l for protocols A, B, C, and D, respectively; NS by ANOVA). After glucagon administration, glucose concentration increased to a similar degree in all protocols (Δ3.6 ± 0.2, 3.3 ± 0.3, 4.04 ± 0.5, and 3.4 ± 0.3 mmol/l in each of the protocols A, B, C, and D, respectively; NS).

**Plasma glucagon concentrations.** Plasma glucagon concentrations...
centrations during the steady-state period were similar on all four study occasions (66 ± 11, 62 ± 5, 66 ± 5, and 59 ± 7 pg/ml for protocols A, B, C, and D, respectively; NS). After the intravenous administration of a glucose bolus (1 mg), the plasma levels rose to >2,000 pg/ml in all four groups.

**Glucose isotopic enrichments and appearance rates.** Isotopic enrichments during the steady-state period 0.5 to 0 h increased with the duration of isotope infusion, independent of tracer (Fig. 2, Table 1). The mean isotopic enrichments (moles % excess) at steady state and the rates of isotope infusions of 13C and 2H2 glucose in each of the four study protocols are provided in Table 1. The calculated glucose Rna’s are illustrated in Fig. 3.

When both isotopes were infused for 2.5 h (protocol A), glucose Rna (µmol · kg⁻¹ · min⁻¹) at steady state was similar for the two isotopes: 11.9 ± 0.4 (1-13C]glucose infusion) and 11.6 ± 0.6 µmol · kg⁻¹ · min⁻¹ ([6,6-2H2]glucose infusion; NS; Fig. 3). Isotopic enrichments were higher after prolonged versus short isotope infusions regardless of isotope (protocols B and C), resulting in lower glucose Rna values: 8.3 ± 0.5 (14.5 h, [1-13C]glucose infusion) vs. 11.2 ± 0.3 µmol · kg⁻¹ · min⁻¹ (2.5 h, [6,6-2H2]glucose infusion; P < 0.01) and 9.2 ± 0.4 (14.5 h, [6,6-2H2]glucose infusion) vs. 11.7 ± 0.3 µmol · kg⁻¹ · min⁻¹ (2.5 h [1-13C]glucose infusion; P < 0.01; Fig. 3). The glucose Rna during a 5-h infusion of [6,6-2H2]glucose (protocol D) was lower (P < 0.01) than that calculated during a 2.5-h infusion of [1-13C]glucose (9.8 ± 0.4 vs. 12.0 ± 0.6 µmol · kg⁻¹ · min⁻¹) and higher than that calculated during the 14.5-h [6,6-2H2]glucose or [1-13C]glucose infusions (protocols C and B; 9.2 ± 0.4 [P < 0.01] or 8.3 ± 0.5 µmol · kg⁻¹ · min⁻¹ [P < 0.01], respectively; Fig. 3).

**Isotope decay curves and t½ of [6,6-2H2]glucose and [1-13C]glucose.** The rate constants (k) of the isotopic enrichment decays after discontinuation of the isotope infusion and the t½ of the isotopes are provided in Table 2. Figure 4 depicts the isotopic enrichments after discontinuation of the infusion through the end of the study (expressed as percentage of the steady-state value), plasma glucose concentrations, and amount of tracer released into the plasma pool after glucagon administration. The decay (k = rate constant × 10⁻³) of the [1-13C]glucose tracer after a 2.5-h infusion (7.1 ± 0.3 was similar (NS) to that after a 14.5-h infusion (6.4 ± 0.2).

The decay of the [1-13C]glucose tracer from the plasma pool after either 2.5 h or 14.5 h of infusion was slower (P < 0.05) than that of the [6,6-2H2]glucose tracer independent of the duration of the [6,6-2H2]glucose infusion (2.5 h, 5 h, or 14.5 h; 8.4 ± 0.3, 8.3 ± 0.3, or 7.6 ± 0.2, respectively). The 2.5-h and 5-h [6,6-2H2]glucose tracer infusions resulted in similar (NS) isotope decays, but they both were faster (P < 0.05) than the [6,6-2H2]glucose tracer infusion after a 14.5-h infusion. From the 14.5-h infusion data, the plasma t½ of [1-13C]glucose was 109 ± 4 min and that of [6,6-2H2]glucose 92 ± 3 min (P < 0.05). The t½ value obtained with the 2.5-h [1-13C]glucose infusion was 99 ± 5 min, and those obtained with the 2.5-h and 5-h [6,6-2H2]glucose infusions were 84 ± 3 min and 84 ± 4 min, respectively (Table 2).

**Glucagon effect on glucose enrichment.** There was no detectable tracer released from glycogen into the plasma pool during the 1 h after administration of glucagon (Fig. 4).

**Lactate enrichment.** The steady-state [13C]lactate enrichments typically were nearly twice as high (1.55 ± 0.1) during the

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**TABLE 1**

<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td>Infusion</td>
<td>%</td>
</tr>
<tr>
<td>A</td>
<td>2.5 h</td>
<td>4.48 ± 0.2</td>
</tr>
<tr>
<td>B</td>
<td>2.5 h</td>
<td>4.58 ± 0.1</td>
</tr>
<tr>
<td>C</td>
<td>14.5 h</td>
<td>5.54 ± 0.2</td>
</tr>
<tr>
<td>D</td>
<td>5 h</td>
<td>5.19 ± 0.2</td>
</tr>
</tbody>
</table>

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14.5-h vs. the 2.5-h [1-13C]glucose infusion (0.85 ± 0.05) in protocols B and C, respectively (P < 0.05).

DISCUSSION

The present studies demonstrate that glucose $R_a$ calculated during a 2.5-h primed constant rate infusion of [1-13C]glucose was overestimated by 35% when compared with that calculated during a 14.5-h infusion of the same glucose tracer. This difference was 26% when the tracer used was [6,6-2H2]glucose. In theory, the overestimation could be due to 1) tracer recycling via glycogen ($2H$ and $13C$) and/or Cori cycle ($13C$) during the prolonged tracer infusion studies, 2) failure to achieve complete equilibration of the tracer with the tracee during the 2.5-h infusion, and 3) isotopic discrimination. From our data, we conclude that glycogen recycling of $13C$ and $2H$ glucose tracers are negligible even after a 14.5-h tracer infusion, whereas recycling, although small, of the $13C$ glucose tracer via the Cori cycle was observed even with a 2.5-h tracer infusion. Because the glucose $R_a$ during simultaneous 2.5-h infusions of [1-13C]glucose and [6,6-2H2]glucose were essentially identical, isotopic discrimination seems unlikely. Thus, failure to achieve complete equilibration of the tracer and tracee during the 2.5-h tracer infusion makes duration of isotope infusion an important factor that affects measures of glucose $R_a$.

Although both the [1-13C]glucose and [6,6-2H2]glucose tracers could theoretically recycle through glycogen via the direct pathway, only the carbon-labeled tracer can recycle back to glucose through the Cori cycle (3–5,10). In the present study, neither the [1-13C]glucose nor the [6,6-2H2]glucose tracer was released into the plasma glucose pool after glucagon infusion, making any significant recycling of tracer from glycogen unlikely.

The [6,6-2H2]glucose tracer decay after 14.5 h or 5 h of infusion was slightly slower (~10%) than that after the 2.5-h infusion. Because no detectable isotope decay curve change was observed after stimulation of glycogenolysis by glucagon infusion, it seems unlikely that this difference would be the result of release of tracer into the plasma space from glycogen. The decay of the [6,6-2H2]glucose tracer was faster than that of the [1-13C]glucose tracer independent of the duration of tracer infusion (Table 2). However, unlike the [6,6-2H2]glucose tracer, the decays of the [1-13C]glucose tracer after 2.5 h or 14.5 h of infusion were similar (NS). This suggests an ongoing contribution of $13C$ label from lactate to glucose during the decay period obscuring a small difference between the 2.5- and 14.5-h infusions as was observed with the [6,6-2H2]glucose tracer. Consistent with this speculation is that the higher lactate $13C$ enrichment was almost twice as high during the prolonged compared with the short [1-13C]glucose infusion. Comparing the isotopic enrichment after the 14.5-h infusion of [6,6-2H2]glucose and [13C]glucose indicates Cori cycle activities of ~10%. This is less than that of Reichard et al. (11) of 16% in normal subjects but greater than that estimated by Tayek et al. (12) of 9%.

TABLE 2
Isotope $t_{1/2}$ (min) and rate constants (k)

<table>
<thead>
<tr>
<th>Protocol</th>
<th>$t_{1/2}$ (min)</th>
<th>Rate constant $\times 10^{-3}$ (k)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.5 h [1-13C]glucose (B)</td>
<td>109 ± 4*</td>
<td>6.4 ± 0.2*</td>
</tr>
<tr>
<td>2.5 h [1-13C]glucose (C)</td>
<td>95 ± 5*</td>
<td>7.1 ± 0.3*</td>
</tr>
<tr>
<td>14.5 h [6,6-2H2]glucose (C)</td>
<td>92 ± 3†</td>
<td>7.6 ± 0.2†</td>
</tr>
<tr>
<td>5 h [6,6-2H2]glucose (D)</td>
<td>84 ± 4</td>
<td>8.3 ± 0.3</td>
</tr>
<tr>
<td>2.5 h [6,6-2H2]glucose (B)</td>
<td>84 ± 3</td>
<td>8.4 ± 0.3</td>
</tr>
</tbody>
</table>

*Significant differences (P < 0.05) between the 14.5-h and 2.5-h [1-13C]glucose infusion compared with the 2.5-h, 5-h, and 14.5-h [6,6-2H2]glucose; †significant differences (P < 0.05) between the 14.5-h [6,6-2H2]glucose compared with the 2.5-h and 5-h [6,6-2H2]glucose infusion.

FIG. 3. Glucose $R_a$ at steady state (~0.5 to 0 h) in all four study protocols: *P < 0.05 within each protocol (A–D).
lar space. Thus, 2.5-h isotope infusion is not sufficient to achieve isotope and substrate equilibration in adults. In addition, this slowly equilibrating pool will complicate any attempt at non–steady-state modeling.

These conclusions in normal adult volunteers are supported by previous studies in patients with type 2 diabetes. In subjects with type 2 diabetes, hepatic glucose production was overestimated when the duration of a [3-3H]glucose infusion was insufficient for complete isotope equilibration, and the error was related to the degree of hyperglycemia (13). Chen et al. (14) demonstrated that at least 4 h of tracer administration was necessary to reach steady state and accurately measure glucose $R_a$ in patients with type 2 diabetes but failed to observe this in normal subjects. They attributed this difference to the expanded plasma glucose pool size and the markedly reduced glucose uptake in the subjects with type 2 diabetes. Using a paired study design, we were able to demonstrate that this applies equally to normal individuals with normal plasma concentrations. Moreover, studies using prolonged isotope infusions in patients with type 2 diabetes could not confirm the increase in hepatic glucose production that was reported in some of the earlier studies using short (90–120 min) infusion periods of [3H]glucose (15,16). These observations are in agreement with the findings of the present study.

In this study, we used a 60-min priming dose of labeled glucose. We and others have used a variety of priming doses ranging from 60 to 100 min (17–19). It is of interest that despite a 90-min prime in the study by Hovorka et al. (3), the change in enrichment from a 120- to a 240-min time point was ~15%, which is nearly identical to the difference that we observed after infusion of isotope for 120 vs. 270 min, i.e., 13% (using a 60-min prime). In addition, these results are consistent with those of Høther-Nielsen et al. (20). These investigators used 3H glucose and provided data from normal control and type 2 diabetic subjects using a fixed prime or a prime adjusted to glycemia. In the normal control subjects, using a 100-min prime, the specific activity increased by 21% between 2.5 and 5 h of the isotope infusion as compared with 13% in our study (using a 60-min prime). In subjects with type 2 diabetes, the corresponding changes were 50% using a fixed 100-min prime and 25% using a prime adjusted to glycemia. Collectively, these results demonstrate that increasing the prime from 60 to 100 min did not overcome the isotopic disequilibrium in normal control subjects during short periods of isotope infusion. In subjects with type 2 diabetes, the use of an adjusted prime was helpful but did not completely overcome isotopic disequilibrium during short periods of isotope infusion. Thus, priming the substrate pool may be useful in approaching an “approximate” steady state during short infusions of glucose tracers but may still result in incomplete equilibration and an underestimation of glucose $R_a$.

Erroneous measures of glucose $R_a$ will also result in propagating errors of measures of gluconeogenesis and glycogenolysis. The most recently described methods to measure gluconeogenesis ([U-13C]glucose [21,22] and [2-13C]glycerol MIDA [23]) and deuterated water with measurement of incorporation of deuterium in glucose carbons 5 and 6 (24) express gluconeogenesis as a fraction of glucose $R_a$. Rates of gluconeogenesis are subsequently calculated as the product of fractional gluconeogenesis and glucose $R_a$. Likewise, measures of glycogenolysis, which are calculated as the difference between glucose $R_a$ and the rate of gluconeogenesis, will also be affected. Similar errors may occur in studies using nuclear magnetic resonance (NMR). NMR provides an objective measure of glycogenolysis, whereas gluconeogenesis is calculated by subtracting glycogenolysis from glucose $R_a$ (measured by GCMS). In some reports using this NMR technique, short duration of tracer infusion was used to measure glucose $R_a$ (2–4 h) (25–27). In these studies (despite using a prime dose corresponding to

![FIG. 4. Glucose concentrations, isotopic enrichments, and tracer release over time, after discontinuation of the infusion and after glucagon. Isotopic enrichment at 0 h was normalized to 100% of the average value obtained during the steady-state period, i.e., between ~0.5 and 0 h.](image-url)
100-min infusion), rates of glucose $R_g$ decreased from 12.6 μmol · kg$^{-1}$ · min$^{-1}$ · day$^{-1}$ at 2 h of isotope infusion to 8.4 μmol · kg$^{-1}$ · min$^{-1}$ · day$^{-1}$ after 4 h of infusion, i.e., 33%, which is in agreement with our results and reemphasizes the importance of using sufficient duration of isotope infusion.

These studies unequivocally demonstrate the importance of the duration of isotope infusion in achieving valid results. Thus, investigators who conduct studies on glucose turnover rates using tracer infusion periods of <4–5 h should be cognizant that their measurements of glucose $R_g$ using steady-state equations may be overestimated, which has to be taken into account when drawing conclusions from their data.

ACKNOWLEDGMENTS

This work is a publication of the U.S. Department of Agriculture/Agricultural Research Service Children’s Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, Houston, TX. These studies were supported in part by grants from the Children’s Nutrition Research Center (USDA/ARS Cooperative Agreement 58-6250-6-001), NIH R01 DK55478, and NIH R01 HD37957. The contents of this publication do not necessarily reflect the views or policies of the U.S. Department of Agriculture, and mention of trade names, commercial products, or organizations does not imply endorsement from the U.S. government.

We thank Shaji Chacko, Cindy Clarke, Dan Donaldson, Kathryn Louie, and Matt Moore for technical assistance and our nurse coordinator Andrea Dotting-Jones and the nursing staff of the Metabolic Research Unit at the Children’s Nutrition Research Center for assistance with the execution of these studies.

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