Use of a novel triple-tracer approach to assess postprandial glucose metabolism

RITA BASU,1 BARBARA DI CAMILLO,2 GIANNA TOFFOLO,2 ANANDA BASU,1 PANKAJ SHAH,1 ADRIAN VELLA,1 ROBERT RIZZA,1 AND CLAUDIO COBELLI2

1Division of Endocrinology, Diabetes, Metabolism, and Nutrition, Department of Internal Medicine, Mayo Clinic and Foundation, Rochester, Minnesota 55905; and 2Department of Electronics and Informatics, University of Padua, Padua 35131, Italy

Use of a novel triple-tracer approach to assess postprandial glucose metabolism. Am J Physiol Endocrinol Metab 284: E55–E69, 2003; 10.1152/ajpendo.00190.2001.—Numerous studies have used the dual-tracer method to assess postprandial glucose metabolism. The present experiments were undertaken to determine whether the marked tracer nonsteady state that occurs with the dual-tracer approach after food ingestion introduces error when it is used to simultaneously measure both meal glucose appearance (Ra meal) and endogenous glucose production (EGP). To do so, a novel triple-tracer approach was designed: 12 subjects ingested a mixed meal containing [1-13C]glucose while [6-3H]glucose and [6,6-2H2]glucose were infused intravenously in patterns that minimized the change in the plasma ratios of [6-3H]glucose to [1-13C]glucose and of [6,6-2H2]glucose to endogenous glucose, respectively. Ra meal and EGP measured with this approach were essentially model independent, since non-steady-state error was minimized by the protocol. Initial splanchnic glucose extraction (ISE) was 12.9 ± 3.4%, and suppression of EGP (EGPS) was 40.3% ± 4.1%. In contrast, when calculated with the dual-tracer one-compartment model, ISE was higher (P < 0.05) and EGPS was lower (P < 0.005) than observed with the triple-tracer approach. These errors could only be prevented by using time-varying volumes different for Ra meal and EGP. Analysis of the dual-tracer data with a two-compartment model reduced but did not totally avoid the problems associated with marked postprandial changes in the tracer-to-tracee ratios. We conclude that results from previous studies that have used the dual-tracer one-compartment model to measure postprandial carbohydrate metabolism need to be reevaluated and that the triple-tracer technique may provide a useful approach for doing so.

AFTER AN OVERNIGHT FAST, the amount of glucose entering the systemic circulation [i.e., endogenous glucose production (EGP)] approximates the amount of glucose leaving the circulation [i.e., glucose disappearance (Ra)]. Under these circumstances, EGP is primarily derived from the liver, with a small contribution coming from the kidney (6, 9, 10, 27). The situation becomes more complex after food ingestion when glucose entering the systemic circulation can originate from both the gut and EGP (9). An alteration in either of these processes can substantially influence glucose tolerance.

In a pioneering series of experiments, Steele et al. (25) introduced a dual-isotope method that enabled simultaneous in vivo measurement of both the systemic rate of appearance of the ingested glucose (Ra meal) and postprandial EGP. This approach utilizes two glucose tracers: one ingested and one infused intravenously. The intravenously infused tracer measures the rates of appearance (Ra) of the ingested tracer and of total glucose (i.e., labeled and unlabeled). Appearance of the ingested glucose is calculated by multiplying the Ra of the ingested tracer by the specific activity (or tracer-to-tracee ratio if a nonradioactive tracer is used) of the meal. Initial splanchnic glucose uptake is calculated by subtracting the portion of the ingested glucose that reaches the systemic circulation from the total amount of glucose ingested. EGP is calculated by subtracting the Ra of the ingested glucose from the total glucose appearance. Glucose Ra is calculated by subtracting the change in glucose mass from the total rate of glucose appearance.

This experimental approach has been used by a large number of investigators to study postprandial glucose metabolism in several species, including rats, dogs, and humans (3, 11, 12, 14–19, 25, 29). Unfortunately, results have not always been consistent. As recently reviewed by Livesey et al. (16), estimates of initial splanchnic glucose uptake have ranged from −4 to 35% (3, 11, 12, 14–19, 29). The pattern of EGP also has varied. Carbohydrate ingestion has been reported to result in either rapid and near-complete suppression of EGP (EGPS; see Refs. 3 and 11), slow but partial EGPS (15, 16, 18), or an apparent initial paradoxical increase followed by a subsequent fall in EGP (12, 14, 29). Although it is possible that these differences are the...
result of biological variation, it is more likely they are because (at least in part) of inadequacies in the model used to calculated turnover during the marked nonsteady state that occurs after carbohydrate ingestion.

Steele recognized that a single-compartment model (1CM) was inadequate during nonsteady state (26). He therefore proposed a constant commonly referred to as the pool fraction (the so-called pool value (p) value) to try to account for incomplete mixing within the single compartment. Over the ensuing years, the proper value for p has remained a matter of debate (1, 16, 20). Even more problematic, experiments have shown that, under non-steady-state conditions, the “correct” value for p varies with time (1, 5, 16). In any case, because a 1CM is assumed, the concentration gradients that are present throughout the glucose system are ignored. These gradients result in errors that could be particularly insidious when their magnitude differs between subjects (e.g., diabetic vs. nondiabetic) or when the size of the gradient is influenced by the conditions being studied (e.g., ingestion of a large vs. a small meal). Two-compartment models (2CM) that allow tracer-to-tracee gradients throughout the body have been proposed in an effort to overcome this problem (17, 21). Recently, Livesey et al. (16) compared the performance of a 2CM with that of a 1CM with pV (where V is the volume of distribution) = 230 ml/kg (i.e., p = 1.0) and found a good agreement in the average profiles of Ra,meal and EGP calculated with the two methods. However, the authors did not have a model-independent measure of Ra and EGP, and this average agreement cannot be considered a proof of validity because both 1CM and 2CM are well known to be affected by non-steady-state errors. These errors can be avoided if the tracer-to-tracee ratio can be maintained constant throughout the experiment. A variant of this approach, commonly referred to as the hot “ging” method, has been used to accurately measure glucose turnover during a glucose clamp (5, 13). This is done by ensuring that the specific activity (or tracer-to-tracee ratio) of the infused glucose is the same as that present in plasma and by concurrently adjusting the tracer infusion rate in a manner that mimics the anticipated pattern of change of EGP. Taylor et al. (28) proposed a similar approach to measure EGP after carbohydrate ingestion. Those experiments used a modification of the conventional dual-tracer approach in which the rate of infusion of intravenous tracer was adjusted to mimic the anticipated pattern of change of EGP. This approach minimized the change in the tracer-to-tracee ratio of endogenous glucose (i.e., the proportion of plasma glucose derived from endogenous sources), thereby permitting more accurate measurement of EGP. However, marked changes in meal and total glucose plasma tracer-to-tracee ratios still occurred, thereby precluding accurate measurement of meal appearance, total glucose appearance, and glucose Ra.

In an effort to circumvent these problems, the present experiments sought to determine whether a novel triple-tracer approach could be used to minimize changes in both meal and endogenous plasma tracer-to-tracee ratios, thereby permitting simultaneous measurement of meal appearance and EGP. Changes in the plasma ratios of the intravenous and ingested tracers were minimized by intravenously infusing [6-3H]glucose in a manner anticipated to mimic the systemic Ra of the [1,13C]glucose contained in a mixed meal. At the same time, changes in the ratio between [6,6-2H2]glucose tracer and endogenous glucose were minimized by infusing [6,6-2H2]glucose in a pattern that mimicked the anticipated pattern of change of EGP. Results were then compared with those observed using the dual-tracer approach in which the intravenously infused was used to trace the Ra of both the ingested [1-13C]glucose and total unlabeled glucose, with EGP being calculated as the difference between the two rates. The dual-tracer data were analyzed using both 1- and 2CM to determine whether increasing the complexity of the model decreases the impact of the nonsteady-state conditions. The accuracy of the dual-tracer method was evaluated both when the [6,6-2H2]glucose infusion rate was varied (accentuating the change in the tracer-to-tracee ratio and therefore creating a “worst-case” scenario) and when the [6,6-2H2]glucose infusion rate was simulated to remain constant (mimicking the conditions generally present with the “conventional” dual-tracer approach).

We present data indicating that the dual-tracer approach analyzed with a 1CM and a fixed value for pV is unable to accurately measure meal appearance, EGP, and glucose Rd after food ingestion. The dual-tracer approach analyzed with a 2CM reduces but does not totally avoid the problems inherent in 1CM. The triple-tracer approach, by minimizing change in the plasma tracer-to-tracee ratios, is essentially model independent and therefore enables more accurate measurement of factors involved in the regulation of postprandial glucose tolerance.

**METHODS**

**Subjects**

After approval from the Mayo Institutional Review Board, 12 healthy subjects (6 women and 6 men, mean age 25 ± 1 yr, height 172 ± 2 cm, weight 77 ± 4 kg, body mass index 25.7 ± 0.8 kg/m2, lean body mass 51 ± 3 kg) gave informed written consent to participate in the study. All subjects were in good health and did not participate in regular vigorous physical activity.

**Experimental Design**

All studies were conducted at the Mayo General Clinic Research Center (GCRC). Subjects consumed a weight maintenance diet (55% carbohydrate, 15% protein, and 30% fat) provided by the GCRC kitchen for 3 days preceding the study. All subjects were admitted at 1600 on the afternoon before study and were given a standard 10 kcal/kg meal that was consumed between 1700 and 1730. No additional food was eaten until the next morning. At ~0600 on the morning...
of study, an 18-gauge cannula was inserted in a retrograde fashion in a dorsal hand vein. The hand was then placed in a heated Plexiglas box (~55°C) to obtain arterialized venous blood samples. Another 18-gauge cannula was inserted in the opposite forearm for tracer infusion. A primed-continuous infusion of [6,6-2H2]glucose (11.84 mg/kg prime; 0.1184 mg·kg⁻¹·min⁻¹ continuous; MassTrace, Woburn, MA) was started at 0700 and continued until the end of the study at 1600.

At 0900 (time 0), a mixed meal (10 kcal/kg, 45% carbohydrate, 15% protein, and 40% fat) consisting of three scrambled eggs, Canadian bacon, 100 ml water, and Jell-O containing [1-13C]glucose was consumed within 15 min. The beaker containing the Jell-O was rinsed twice with 20 ml water, and the rinse solution was consumed. To prepare the Jell-O, 1.2 g/kg body wt dextrose was dissolved in 200 ml water by gentle heating. After cooling to room temperature, sufficient [1-13C]glucose was added to achieve an enrichment of ~4%. After thorough mixing, an aliquot was removed for analysis of [1-13C]glucose enrichment by gas chromatography-mass spectrometry (GC/MS). The dextrose solution containing the [1-13C]glucose was then warmed gently, and 5 g sugar-free gelatin (Knox unflavored gelatin; Nabisco, East Hanover, NJ) and 1 g sugar-free orange-flavored Kool-Aid (Kraft General Foods, White Plains, NY) were added. The mixture was allowed to solidify overnight in the refrigerator.

An infusion of [6-3H]glucose was started at time 0 (i.e., with the first bite), and the rate varied to mimic the anticipated R of the [1-13C]glucose contained within in the meal (Fig. 1). At the same time, the rate of infusion of [6,6-2H2]glucose was altered so as to approximate the anticipated pattern of fall in EGP. Blood was sampled from the arterialized venous site at ~10, 20, 30, 40, 50, 60, 75, 90, 120, 150, 180, 210, 240, 260, 280, 300, 360, and 420 min.

Analytical Techniques

Plasma samples were placed on ice, centrifuged at 4°C, separated, and stored at ~20°C until assay. Plasma glucose concentration was measured using a glucose oxidase method (YSI, Yellow Springs, OH). Plasma insulin concentration was measured using a chemiluminescence assay with reagents obtained from Beckman Coulter (Access Assay; Beckman Coulter, Chaska, MN). Body composition was measured using dual-energy X-ray absorptiometry (DPX scanner; Lunar, Madison, WI). Plasma [6-3H]glucose specific activity was measured by liquid scintillation counting, as previously described (23). Plasma enrichment of [1-13C]glucose and [6,6-2H2]glucose was measured using GC/MS (Thermoquest, San Jose, CA) to simultaneously monitor the C-1 and C-2 and C-3 to C-6 fragments, as described by Beylot et al. (4). Pilot experiments in five subjects established that, under the conditions of the present experiment, enrichment of 13C resulting from carbon cycling is below the limit of detection with this technique.

Calculations

To arrive at the formulas for calculating the fluxes, we first derive the tracer and tracee glucose concentrations in plasma from the measurements.

Glossary

To do so, we used the following definitions:

- **Tracee** glucose at natural composition, coming from ingested glucose and EGP
- **Tracer1** [1-13C]glucose above natural abundance
- **TracerII** [6,6-2H2]glucose tracer, consisting primarily of [6,6-2H2]glucose (tracer purity is 92%), a minor amount of [6,6-2H1-1H1]glucose, and a negligible amount of [6,6-2H2]glucose
- **TracerIII** [6-3H]glucose tracer
- **Gnat** concentration of tracee in plasma (μmol/ml)
- **G13C** concentration of tracer1 in plasma (μmol/ml)
- **G2H** concentration of tracerII in plasma (μmol/ml)
- **G3H** concentration of tracerIII in plasma (μmol/ml)
- **F2H** rate of intravenous infusion of tracer II (dpm/min)
- **F3H** rate of intravenous infusion of tracer III (dpm/min)
- **G** plasma glucose concentration measured in the arterialized hand vein samples (μmol/ml)
- **MR13C** ratio of [1-13C]glucose to [1-12C]glucose in plasma
- **MR13C_meal** MR13C measured in the meal
- **MR2H** ratio of [6,6-2H2]glucose to [6,6-2H2]glucose in plasma

Measurements and tracer and tracee glucose concentrations. MR13C is measured in the C-1 and C-2 fragments. Tracer1 contributes to [1-13C]glucose only; tracerII and tracerIII, having the carbon atom at natural composition, contrib-
ute to [1-12C]glucose with 1/(1 + MR13c_nat) equal to 98.9% of their mass and to [1-13C]glucose with the remaining MR13c_nat/ (1 + MR13c_nat) equal to 1.1%

\[
\text{tracer}^1 + (\text{tracee} + \text{tracer}^\text{II})
\]

\[
MR_{13c} = \frac{\text{tracer}^1 \times MR_{13c,\text{net}}/(1 + MR_{13c,\text{net}})}{(\text{tracer} + \text{tracee} + \text{tracer}^\text{II})/(1 + MR_{13c,\text{net}})}
= \frac{\text{tracer}^1}{(\text{tracer} + \text{tracee} + \text{tracer}^\text{II})/(1 + MR_{13c,\text{net}})} + MR_{13c,\text{net}} \quad (I)
\]

where MR_{13c,\text{net}} represents the ratio between 13C and 12C in natural glucose, equal to 0.011. Thus MR_{13c} above natural, which is determined from the mass-to-charge ratio (m/z) of 161 to 160 corrected for natural abundance of all atoms, is related to tracer^1, tracer^II, and tracee by the following formula

\[
MR_{13c} - MR_{13c,\text{nat}} = \frac{\text{tracer}^1 \times MR_{13c,\text{net}}/(1 + MR_{13c,\text{net}})}{(\text{tracer} + \text{tracee} + \text{tracer}^\text{II})/(1 + MR_{13c,\text{net}})}
= \frac{G_{13c}}{G_{\text{nat}} + G_{2H} - (1 + MR_{13c,\text{net}})} \quad (2)
\]

Similarly, MR_{13c,\text{meal}} is proportional to the ratio between tracer^1 and natural glucose in the meal (ttr\text{meal})

\[
MR_{13c,\text{meal}} - MR_{13c,\text{nat}} = \frac{\text{tracer}^1 \times (1 + MR_{13c})}{\text{tracee} \times (1 + MR_{13c})} = \frac{\text{tracer}^1}{(1 + MR_{13c})} \quad (3)
\]

MR_2H is measured in the C-3 to C-6 fragment and is determined from the m/z 321–319 by correcting for natural abundance of all atoms. It quantifies 92% of tracer^II divided by tracer plus tracer^1

\[
MR_{2H} = \frac{\text{tracer}^\text{II} \times 0.92}{\text{tracer}^1} \quad (4)
\]

Total plasma glucose concentration (G) measures the sum of the concentration of tracee, tracer^1, and tracer^II in plasma

\[
G = G_{\text{nat}} + G_{13c} + G_{2H}
\]

From Eqs. 2, 4, and 5, G_{13c}, G_{2H}, and G_{\text{nat}} can be quantified from the measurements

\[
G_{13c} = \frac{(MR_{13c} - MR_{13c,\text{nat}})}{1 + MR_{13c}} 
G_{2H} = \frac{MR_{2H} \times 0.92}{G_{\text{nat}} + G_{13c}} \quad (6)
G_{\text{nat}} = G - G_{13c} - G_{2H} \quad (7)
\]

The concentration of plasma glucose derived from EGP, (G_{\text{end}}) can be calculated by subtracting the contribution of ingested natural glucose (which is proportional to G_{13c,\text{nat}} as measured by ttr\text{meal}) from the total plasma glucose concentration (i.e., G_{\text{nat}})

\[
G_{\text{end}} = G_{\text{nat}} - G_{13c}/ttr_{\text{meal}} \quad (9)
\]

Calculation of postprandial rates of EGP, appearance of meal-derived glucose, and glucose Ra. Postprandial rates of EGP, appearance of meal-derived glucose, and glucose Ra were calculated using either the triple-tracer or dual-tracer methods.

**Triple-tracer method**

Rates of turnover are calculated using three separate models. Each uses three tracers. In each, [6-3H]glucose traces the systemic Ra of the [1-13C]glucose contained in the meal (referred to as Ra_{13c}), whereas [6,6-2H2]glucose traces the Ra of endogenously produced glucose. The ratio of the plasma concentration of [6-3H]glucose to [1-13C]glucose (i.e., the G3H-to-G13C ratio) is used to calculate Ra_{13c}, and the ratio of the plasma concentration of [6,6-2H2]glucose to the plasma concentration of endogenous glucose (i.e., the G2H-to-G_{\text{end}} ratio) is used to calculate EGP.

**Triple-tracer method calculated with a tracer-to-tracee "clamp" formula.** Assuming a “perfect” clamp of the plasma tracer-to-tracee ratio (i.e., variations of G_{2H}/G_{13C} ratio are negligible), Ra_{13c} equals the infusion rate of the tracer (i.e., [6-3H]glucose) divided by the plasma tracer-to-tracee ratio (i.e., G_{2H}/G_{13C} ratio).

\[
Ra_{13c} = \frac{F_{3H}}{G_{3H}/G_{13C}} \quad (10)
\]

The systemic Ra of ingested glucose (Ra\text{meal}) is calculated by multiplying Ra_{13c} by the meal enrichment [i.e., the ratio of total glucose (tracee + tracer^1) to tracer^1 in the meal]

\[
Ra\text{meal} = Ra_{13c} \times \left(\frac{\text{tracer}^1}{\text{tracee}}\right)_{\text{meal}} = Ra_{13c} \times \left(1 + \frac{1}{ttr_{\text{meal}}} + 1\right) \quad (11)
\]

Initial splanchnic glucose extraction (ISE) is then calculated as

\[
D - \int_{0}^{ttr_{\text{meal}}} Ra\text{meal}(t)dt
ISE = \frac{D}{\int_{0}^{ttr_{\text{meal}}} D} \times 100 \quad (12)
\]

where D is the total amount of glucose ingested, and the amount of the ingested glucose that reaches the systemic circulation is equal to the area under the curve of Ra\text{meal} over the 7 h of study; t is time.

EGP is calculated by dividing the infusion rate of F_{2H} by the plasma ratio G_{2H}/G_{\text{end}}

\[
EGP = \frac{F_{2H}}{G_{2H}/G_{\text{end}}} \quad (13)
\]

Suppression of EGP (EGPS) is calculated by normalizing the area below basal to the basal area

\[
\text{EGPS} = \left[EGP(t) - EGP_{b}\right]dt \times \left[EGP_{b} \times 420\right] \times 100 \quad (14)
\]

where EGP_{b} is the basal rate of EGP. Although the estimation of Ra\text{meal} and EGP is model independent, that of the rate of glucose Ra requires the assumption of a model. We have calculated Ra_{13c} by assuming a ICM (see Eq. 19 below).

**Triple-tracer method calculated with a ICM.** This model is based on a single-compartment description of the system and was introduced in Ref. 22. As detailed in APPENDIX A, an expression for Ra_{13c} is derived by first expressing the disappearance rate parameter k_{01} from the mass balance equation of [6-3H]glucose tracer

\[
k_{01,3H} = -\frac{dG_{3H}/dt}{G_{3H}/pVG_{3H}} \quad (15)
\]
where $V$ is the glucose volume of distribution and $p$ is the pool fraction, and then by using Eq. 15 in the mass balance equation of $[1-^{13}C]$glucose tracer

$$R_{a} = \frac{F_{g1}}{G_{a}^{13C}} - \frac{pV_{G}}{G_{a}^{13C}} \times \frac{d(G_{a}^{13C}/G_{a}^{13C})}{dt} (16)$$

By applying the same rationale to $[6,6-^{2}H_{2}]$glucose and endogenous glucose, expressions of the disappearance rate parameter of $[6,6-^{2}H_{2}]$glucose tracer $k_{01,2H}$ (min$^{-1}$) and EGP are derived

$$k_{01,2H} = -\frac{dG_{2H}/dt}{G_{2H}} + \frac{F_{2H}}{pV_{G}} (17)$$

$$EGP = \frac{F_{2H}}{G_{2H}} - \frac{pG_{end}}{d(G_{2H}/G_{end})/dt} (18)$$

$R_{m, meal}$, ISE, and EGP are calculated using Eqs. 11, 12, and 14. $R_{d}$ (pmol/min) is calculated as

$$R_{d} = (R_{m, meal} + EGP + F_{2H}) - pV \frac{dG}{dt} (19)$$

$V$ was initially assumed to equal 200 ml/kg body wt and $p$ to equal 0.65 ($pV = 130$ ml/kg). Values for $pV$ were then subsequently varied from 130 to 160 (which represents a total glucose distribution volume of 250 ml/kg, according to Ref. 7 multiplied by a $p$ equal to 0.65) to 230 (as proposed in Ref. 16) ml/kg to examine the dependence of dual- and triple-tracer approaches on the assumed glucose $V$.

**Triple-tracer method calculated with a 2CM.** This model (22) assumes a two-compartment description of the system with a time-varying $R_{a}$ of glucose from the accessible compartment ($k_{01}$) and constant rate parameters between the accessible and the peripheral compartments ($k_{21}$, $k_{12}$). As derived in Appendix A, expressions for the disappearance rate parameter $k_{01}$ of the $[6-^{2}H]$glucose tracer and $R_{a}$ are

$$k_{01,3H} = -\frac{dG_{3H}/dt}{G_{3H}} + \frac{F_{3H}}{V_{G}} - k_{21} + \frac{k_{12}Q_{2,3H}}{V_{G}G_{3H}} (20)$$

$$R_{a} = \frac{F_{3H}}{G_{3H}/G_{13C}} - \frac{V_{G}G_{13C}}{G_{3H}/G_{13C}} \times \frac{d(G_{13C}/G_{13C})}{dt} + k_{12} \left( \frac{Q_{2,3H}}{G_{2H}/G_{13C}} - Q_{2,13C} \right) (21)$$

where $V_{G}$ is glucose volume of distribution of the accessible pool (assumed to equal 130 ml/kg), $k_{21}$ and $k_{12}$ are constant rate parameters between the peripheral and the accessible compartment (assumed equal to 0.05 and 0.07 min$^{-1}$, respectively), and $Q_{2,13C}$ and $Q_{2,3H}$ are the amounts of $[1-^{13}C]$glucose and $[6-^{2}H]$glucose tracer present in the peripheral compartment (determined by integrating model equations as detailed in Appendix A).

Similarly, EGP and the time-varying disappearance rate parameter $k_{01}$ of $[6,6-^{2}H_{2}]$glucose tracer are expressed as

$$k_{01,2H} = -\frac{dG_{2H}/dt}{G_{2H}} + \frac{F_{2H}}{V_{G}} - k_{21} + \frac{k_{12}Q_{2,2H}}{V_{G}G_{2H}} (22)$$

$$EGP = \frac{F_{2H}}{G_{2H}/G_{end}} - \frac{V_{G}G_{end}}{d(G_{2H}/G_{end})/dt} + k_{12} \left( \frac{Q_{2,2H}}{G_{2H}/G_{end}} - Q_{end} \right) (23)$$

where $Q_{2,2H}$ and $Q_{2,3H}$ are the amounts of $[6,6-^{2}H_{2}]$glucose tracer and endogenous glucose in the peripheral compartment determined by integrating model equations.

$R_{m, meal}$, ISE, and EGP are calculated as indicated before by using Eqs. 11, 12, and 14. $R_{d}$ can be expressed as

$$R_{d} = (R_{m, meal} + EGP + F_{2H}) - \frac{V}{t_{d}} F_{G} - k_{21} V_{G} + k_{12} Q_{2} (24)$$

where $Q_{2}$ is the amount of glucose in the peripheral compartment to be evaluated by integrating model equations.

**Worst-Case Dual-tracer Method.** In the present experiment, the infusion rate of $[6,6-^{2}H_{2}]$glucose was varied in a pattern that approximated the anticipated pattern of change of EGP. Although this minimized the change in the ratio of $[6,6-^{2}H_{2}]$glucose to endogenous glucose concentration (used to calculate EGP with the triple-tracer method), it accentuated the change in the ratio of the plasma $[6,6-^{2}H_{2}]$glucose to total glucose (used to calculate total glucose appearance with the dual-tracer method) and the ratio of plasma $[6,6-^{2}H_{2}]$glucose to $[1-^{13}C]$glucose (used to calculate meal appearance with the dual-tracer method). This analysis therefore is referred to as the worst-case dual-tracer method. $R_{a}$ was calculated using the ICM equation similar to Eq. 16, with $F_{2H}$ and $G_{2H}$ in place of $F_{3H}$ and $G_{3H}$, respectively. $R_{m, meal}$ and ISE were calculated as for the triple-tracer method by using Eqs. 11 and 12, respectively.

The total $R_{d}$ for glucose was calculated by applying an equation similar to Eq. 18, with $G$ in place of $G_{end}$. EGP was calculated by subtracting both $R_{m, meal}$ and the infusion rate of $[6,6-^{2}H_{2}]$glucose from $R_{d}$. EGP was calculated using Eq. 14.

The 2CM also was used to calculate turnover. $R_{a}$ was calculated by applying the 2CM equation similar to Eq. 21, with $F_{2H}$ and $G_{2H}$ in place of $F_{3H}$ and $G_{3H}$, respectively. $R_{m, meal}$ and ISE were calculated as for the triple-tracer method, by applying Eqs. 11 and 12, respectively. $R_{d}$ was calculated by applying the 2CM equation similar to Eq. 23, with $G$ in place of $G_{end}$. EGP was then calculated by subtracting $R_{m, meal}$ and the infusion rate of $[6,6-^{2}H_{2}]$glucose from total $R_{d}$. EGP was calculated using Eq. 14.

In contrast to the triple-tracer approach, the marked variation in the ratios of $G_{2H}$ to $G_{13C}$ and $G_{2H}$ to $G$ that occur with the dual-tracer approach precluded the use of the clamp formula (CF) to calculate turnover.

**Data Analysis.** All data are expressed as the means ± SE. Rates of glucose turnover are expressed as micromole per kilogram lean body mass. Values obtained from −30 to 0 min (i.e., before the meal) were considered as basal.

The time derivatives of the tracer-to-tracee ratios appearing in both 1CM and 2CM (Eqs. 15–24) have been calculated by using a new algorithm based on a stochastic regularization method (Ref. 8). Traditional methods evaluate time derivatives using a two-step process, i.e., the data are first smoothed followed by numerical estimation of the time derivatives. The new algorithm, by simultaneously performing both data regularization the time-derivative calculations, provides an estimate of the time derivatives on a uniform, arbitrarily fine grid. However, because the tracer infusions used in the present experiments were changed in a stepwise manner at times that coincided with some of the sampling times, the derivative estimation at these times can be prob-
lematic, since tracer concentrations, and thus tracer-to-tracee ratios, can have a discontinuous time derivative. To avoid this problem, 1CM and 2CM calculations were performed at midpoints between sampling times.

To avoid oversmoothing during the initial few minutes after meal ingestion, the initial G_{3H}-to-G_{13C} ratio, at time 10 min, was not used to calculate R_a meal in the triple-tracer analyses. This time point was excluded either because measurements were not available (G_{13C} and G_{3H} were below the limit of detection in 3 and 6 subjects, respectively) or because the ratio was very high and of uncertain reliability, as indicated by an error propagation analysis, in the remaining subjects. Based on the same rationale, G_{2H}-to-G_{13C} ratios at time 10 and 15 min were not used in the calculation of R_a meal in the dual-tracer analyses.

Areas under curves appearing in Eqs. 12 and 14 were calculated using the trapezoidal rule. Area under the R_a meal curve was calculated by assuming that the systemic R_a of ingested glucose was zero until plasma G_{13C} first became detectable. Triple-tracer R_d and dual-tracer EGP (both of which require knowledge of R_a meal) were calculated starting from the time when R_a meal was first reliably detected.

The Wilcoxon’s signed-rank test was used to determine the statistical significance of differences. A P value of <0.05 was considered to be statistically significant.

RESULTS

Plasma Glucose, Glucose Tracer, and Insulin Measurements

Plasma glucose concentrations averaged 4.8 ± 0.1 mmol/l before meal ingestion and peaked 40 min after meal ingestion at 8.9 ± 0.4 mmol/l (Fig. 2). Glucose concentrations returned to values that no longer differed from basal by ~180 min. Insulin concentration averaged 21 ± 3 pmol/l before meal ingestion and peaked ~30 min after meal ingestion at 494 ± 77 pmol/l. As with glucose, insulin concentrations returned to basal values by ~180 min.

MR_{13C} in plasma averaged a maximum value of 3.45 ± 0.3% above basal ~120 min after meal ingestion and then declined to 1.38 ± 0.14% above basal at the end of the experiment. MR_{2H} in plasma averaged 4.20 ± 0.16% before meal ingestion, it declined to a minimum value of 0.68 ± 0.05% at ~150 min after meal ingestion, and then increased to 2.92 ± 0.15% at the end of the experiment. [6-3H]glucose tracer concentration peaked at 2,232 ± 142 dpm/ml at ~50 min after meal ingestion and then decreased to 1,115 ± 102 dpm/ml on the average.

Plasma Glucose Tracer-to-Tracee Ratios

The triple-tracer approach uses [6,6-2H_2]glucose to trace the R_a of endogenously produced glucose and [6-3H]glucose to trace the R_a of the ingested glucose. To do so, the intravenous infusion rate of [6,6-2H_2]glucose tracer was varied to mimic the anticipated pattern of change of EGP, whereas the intravenous infusion rate of [6-3H]glucose was varied to mimic the anticipated R_a of the ingested [1-13C]glucose. Figure 3, left, shows that this approach minimized the postprandial change in the plasma ratio of [6,6-2H_2]glucose tracer to endogenous glucose. On the other hand, as shown in Fig. 3, Fig. 2. Mean glucose and insulin concentrations, the plasma ratio of [1-13C]glucose to [1-12C]glucose (above natural), the plasma ratio of [6,6-2H_2]glucose to [6,6-1H_2]glucose, and the [6-3H]glucose concentration measured in plasma during the time course of the experiment. See Glossary for definitions.
there was a decrease (∼2-fold) in the plasma ratio of [6-3H]glucose to [1-13C]glucose during the first 15–40 min after meal ingestion, with modest changes thereafter.

More pronounced variations were obtained when the [6,6-2H2]glucose was used to trace the Ra of both unla-
beled glucose and [1-13C]glucose. The rate of infusion of [6,6-2H2]glucose was varied as part of the triple-tracer protocol. Because this created even more marked changes in the tracer to tracee ratios, it is referred to as a worst-case scenario for the dual-tracer approach. As is evident from Fig. 3, right, this resulted in an ∼10-fold variation in the plasma [6,6-2H2]glucose tracer to ingested [1-13C]glucose ratio (Fig. 3, bottom right) and an ∼6-fold variation in the ratio of [6,6-
2H2]glucose tracer to total glucose concentration (Fig. 3, top right).

Glucose Fluxes Calculated Using the Triple-Tracer Method

The systemic Ra of the ingested glucose calculated with 1CM and 2CM and the triple-tracer approach were almost superimposable. Calculation with CF (which assumes complete tracer/tracee steady state) provided a similar pattern; however, rates were slightly lower than with either the 1CM or 2CM until ∼70 min and slightly higher thereafter (Fig. 4A). ISE did not differ when calculated with 1CM, 2CM, or CF (12.9 ± 3.4 vs. 13.7 ± 3.6 vs. 12.2 ± 3.5%), and the individual values were strongly correlated (CF vs. 1CM: r = 0.94, P < 0.0001; CF vs. 2CM: r = 0.84, P < 0.0005; 1CM vs. 2CM: r = 0.97, P < 0.0001).

The pattern of change of postprandial EGP was influenced by the choice of the formula used for its calculation (Fig. 4B). All three formulas showed a similar rate of suppression. However, the rise toward basal rates was faster with 2CM than 1CM, which in turn was faster than that observed with CF. The pattern tended to reverse at ∼225 min when the rise was faster with CF than the other two formulas. However, although the patterns differed slightly, the degree of suppression was similar with 1CM, 2CM, and CF (40.3 ± 4.1 vs. 38.6 ± 4.4 vs. 37.8 ± 4.1%). In addition, the individual values were strongly correlated as follows: r = 0.99, P < 0.0001 for 1CM vs. 2CM; r = 0.99, P < 0.0001 for CF vs. 1CM; and r = 0.97, P < 0.0001 for CF vs. 2CM.

Calculation of turnover using the 1CM, 2CM, and CF indicated a similar pattern of change of glucose Ra after meal ingestion (Fig. 4C). However, 2CM suggested a slightly less rapid rise and slightly less rapid fall in glucose Ra than did 1CM and CF. The postprandial increment in glucose Ra, measured as the area above basal, did not differ for 1CM, 2CM, and CF (5.6 ± 0.4 vs. 5.6 ± 0.3 vs. 5.9 ± 0.4 mmol·kg⁻¹·7 h⁻¹), with values for each individual being strongly corre-
lated (r = 0.98, P < 0.0001 for 1CM vs. 2CM; r = 0.97, P < 0.0001 for CF vs. 1CM; and r = 0.91, P < 0.0001 for CF vs. 2CM).

Glucose Fluxes Calculated Using the Worst-Case Dual-Tracer Method

As is evident from Fig. 3, ingestion of a carbohydrate-containing meal and variation of the [6,6-
2H2]glucose infusion rate resulted in a rapid fall in both the plasma [6,6-2H2]glucose-to-[1-13C]glucose ra-
tio and the [6,6-2H2]glucose-to-total plasma glucose ratio. Because of wide variation in the G2H-to-G13C ratio (see Fig. 3, bottom right), rates of meal appearance could not be calculated reliably with either model until at least 25 min after meal ingestion.
With the 1CM, appearance of ingested glucose measured with the dual-tracer approach peaked later (34 ± 2 vs. 23 ± 1 min; \( P < 0.005 \)) and at a lower rate (52.0 ± 5.4 vs. 84.3 ± 4.2 \( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \); \( P < 0.005 \)) than did that measured with the triple-tracer approach (Fig. 5A, top). Appearance of ingested glucose measured with the dual-tracer approach and 2CM peaked slightly later (37 ± 3 vs. 26 ± 2 min; \( P < 0.01 \)) than did that measured with the triple-tracer approach and 2CM, but the rate was not significantly different (81.2 ± 6.6 vs. 85.8 ± 4.3 \( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \); Fig. 5B, top). The dual-tracer approach and 1CM indicated that less (\( P < 0.005 \)) meal-derived glucose entered the systemic circulation during the 7 h of study than did the triple-tracer approaches (6.3 ± 0.5 vs. 8.1 ± 0.5 mmol \cdot \text{kg}^{-1} \cdot \text{h}^{-1} ). IS were calculated with the dual-tracer approach and 1CM was thus higher (\( P < 0.005 \)) than that calculated with the triple-tracer approach (32.9 ± 4.4 vs. 12.9 ± 3.4%). In contrast, neither the total amount of meal-derived glucose that entered the systemic circulation during the 7 h of study (7.9 ± 0.6 vs. 8.1 ± 0.5 mmol \cdot \text{kg}^{-1} \cdot \text{h}^{-1} ) nor IS (15.1 ± 5.3 vs. 13.7 ± 3.6%) differed with dual-tracer 2CM and triple-tracer 2CM approaches. However, the correlation of the individual values as determined in the two cases was only modest (\( r = 0.64; P < 0.029 \)).

The pattern of change of postprandial EGP also differed with the dual-tracer approach and 1CM vs. triple-tracer approach and 1CM (Fig. 5A, bottom). EGP calculated with the dual-tracer approach and 1CM was lower (\( P < 0.005 \)) during the first 25 min after meal ingestion than that calculated with the triple-tracer approach. Both methods showed a comparable rise toward basal from 70 min onward. Percent suppression below basal was lower (\( P < 0.005 \)) with the dual- than triple-tracer approach (34.8 ± 4.5 vs. 40.3 ± 4.1%). In contrast, the pattern of change of postprandial EGP was similar from 25 min onward (i.e., the first time when EGP can be calculated with the dual-tracer approach) with the dual-tracer 2CM and triple-tracer approaches (Fig. 5B, bottom); the postprandial nadir was the same (69 ± 16 min), with a comparable subsequent rate of rise toward basal. The degree of EGPS was also the same (42.9 ± 3.8 vs. 38.6 ± 4.4%), and the individual rates of suppression were well correlated (\( r = 0.982, P < 0.0001 \)).

**Fig. 4. Systemic rate of appearance of meal-derived glucose (A), EGP (B), and glucose disappearance (C) calculated with the triple-tracer approach using the clamp formula (CF) and one (1CM) and two (2CM)-compartment models.**

Fractional Clearance of Tracer Calculated with the 1CM and 2CM

Infusion of a tracer allows calculation of the clearance rate of the tracer from which \( R_a \) and \( R_d \) can be derived. Two tracers were infused simultaneously with the triple-tracer approach (i.e., \([6,6-2H_2]\text{glucose and}[6-3H]\text{glucose}\)). This enabled the time course of the fractional disappearance rate parameter \( k_01 \) to be determined for each tracer using Eqs. 20 and 22 for the 2CM approach. When calculated with the 2CM approach, the time course of \( k_01 \) for \([6,6-2H_2]\text{glucose and}[6-3H]\text{glucose}\) was very similar, particularly from 35 min onward (Fig. 6A). In contrast, the time course of \( k_01 \) for \([6,6-2H_2]\text{glucose and}[6-3H]\text{glucose}\) differed markedly when calculated with the 1CM approach (Fig. 6B), indicating a failure of this model to accurately describe the postprandial tracer (and therefore tracee) kinetics.

The \( V \) of the 1CM calculation was assumed to be constant and equal to 130 ml/kg. By equating Eqs. 16 and 17 and solving for \( V \), one can determine the value of \( V \) at each time point that is required for the pattern of change of \( k_01 \) to be the same for both of the intravenously infused tracers. As shown in Fig. 6C, the requisite value of \( V \) varies over time, ranging from 125 to 293 ml/kg, indicating the inability of the 1CM and any fixed \( V \) to accurately describe postprandial tracer (and therefore tracee) kinetics.
Role of p and V in 1CM Calculations

Previous studies have used p values ranging from 0.65 to 1 and pV values ranging from 130 to 230 ml/kg. Recently, Livesey et al. (16) have suggested that the use of a V of 230 ml/kg (i.e., p = 1) enables accurate estimation of postprandial turnover. We therefore determined the effect of varying pV from 130 to 160 to 230 ml/kg on meal appearance, EGP, and glucose Rd calculated using the dual- and triple-tracer approaches. As is evident in Fig. 7, meal appearance, EGP, and glucose Rd calculated with the triple-tracer approach were essentially independent of the value assumed for pV. In contrast, the value assumed for pV had a marked effect on turnover calculated with the worst-case dual-tracer approach. The apparent Ra of the ingested glucose increased and glucose Rd decreased as pV was increased from 130 to 160 to 230 ml/kg (Fig. 8, A and C). Similarly, the apparent pattern of EGPS was dependent on the value of pV (Fig. 8B), with EGP appearing to paradoxically increase during the first 25 min with respect to basal value when pV was assumed to equal 230 ml/kg.

Because no single pV appeared to be adequate when used with the 1CM dual-tracer approach, we calculated the time-varying pV values required to derive glucose fluxes equal to those obtained with triple-tracer 2CM. As is evident in Fig. 9, the resultant pV values for meal appearance ranged from 166 to 314 ml/kg (mean 204 ml/kg), for EGP from 60 to 135 ml/kg (mean 122 ml/kg), and for glucose Rd from 70 to 319 ml/kg (mean 196 ml/kg).

DISCUSSION

The rate and pattern of change of plasma glucose concentration is determined by the difference between the amount of glucose entering and the amount of glucose leaving the systemic circulation. After carbohydrate ingestion, glucose entering the circulation can either be derived from the meal or produced by the liver (and perhaps the kidney). The so-called dual-tracer approach has been used extensively to study the regulation of these processes under a variety of conditions (3, 11, 12, 14–19, 25, 29). Unfortunately, accurate measurement of meal appearance and EGP after food ingestion is difficult because both vary with time. This creates a non-steady-state situation that necessitates the use of a model to describe the glucose system. Data from the present study indicate that a 1CM and a fixed V is not adequate when the tracer-to-tracee ratio exhibits large variations as in the present study when only the [6,6-2H2]glucose tracer is used to evaluate both meal appearance and EGP (worst case dual tracer). Use of a 2CM substantially improves estimates of these fluxes. However, this approach is still limited, since the results depend on the reliability of the assumed model. The triple-tracer approach, by minimizing postprandial changes in meal and endogenous plasma tracee-to-tracer ratios, is essentially model independent, thereby enabling reliable assessment of meal appearance and EGP.

The triple-tracer approach used in the present studies sought to minimize the change in the plasma ratios of [6-3H]glucose to [1-13C]glucose and [6,6-2H2]glucose to the endogenous glucose concentration. To do so, the
[6-3H]glucose had to be infused intravenously in a manner that precisely matched the systemic Ra of the ingested [1-13C]glucose. This obviously is impossible, since the Ra of [1-13C]glucose is influenced by multiple factors, including the rate of gastric emptying, the rate of glucose absorption, and the rate of hepatic glucose uptake. Although these processes almost certainly vary from individual to individual, on the average, they were sufficiently consistent so that a [6-3H]glucose infusion profile mimicking [1-13C]glucose appearance could be devised following a few pilot experiments. However, the [6-3H]glucose-to-[1-13C]glucose ratio still varied among subjects during the first 10–15 min after meal ingestion. This occurred for at least two reasons. First, neither tracer was present before the meal was eaten, and thus the ratio between [6-3H]- and [1-13C]glucose tracer concentrations either started from zero or infinity, depending on whether [1-13C]glucose appeared before or after [6-3H]glucose.

The uncertainty during the first 10–15 min after meal ingestion potentially could be avoided if both tracers were infused before meal ingestion in a ratio approximating that anticipated to be present in plasma after meal ingestion. However, this would further increase the complexity of an already complicated protocol. Second, glucose absorption likely is more variable immediately after eating than it is once the stomach begins emptying nutrients at a relatively constant rate in the duodenum. Therefore, it is more difficult to guess how much [6-3H]glucose needs to be infused in the first few minutes after food ingestion. On the other hand, as is evident in Fig. 3, the change in the ratio of [6-3H]glucose to [1-13C]glucose over this interval was approximately fivefold lower than the change in the plasma enrichment of [6,6-2H2]glucose over the same interval. This led to a lower error in measurement of meal appearance with the triple- than the dual-tracer approach. Ideally, meal appearance should be measured.
accurately immediately after the start of a meal. The present data indicate that, even with the triple-tracer approach, meal appearance only can be reliably estimated from \(t > 15\) min onward. However, this is not a major limitation, since gastric emptying and therefore meal appearance is likely to be minimal before this time.

Previous estimates of ISE obtained using the conventional dual-tracer method have varied widely, ranging from 0 to 45% (3, 11, 12, 14–19, 29). Although differences in the type of subject studied, composition of food ingested, and metabolism of the tracers used may have contributed to this variability, inaccuracy introduced by nonsteady state and the volume correction factor assumed for “p” in Steele’s 1CM likely has been a major factor (26). Use of the triple-tracer approach indicates that ISE averaged \(-13\%\) in the present experiments. Recent experiments by ourselves (2) and others (17) using the hepatic catheter technique combined with a multiple-tracer approach indicate that splanchnic clearance of enterally infused glucose ranges from \(-15\) to \(-25\%\) in the presence of prolonged steady-state hyperglycemia and hyperinsulinemia. However, it is currently unknown whether splanchnic clearance of glucose in the presence of prolonged and sustained hyperglycemia and hyperinsulinemia is the same as that which occurs after meal ingestion when glucose and insulin concentrations are continuously changing. In addition, it is not known whether the gut and liver (the main contributors to splanchnic glucose uptake) clear the same amount of glucose in the presence of additional enteral nutrients (i.e., fat and protein).

The triple-tracer approach uses the ratio between \([6,6-{2H}_2]\)glucose and endogenous glucose to calculate EGP. The endogenous glucose concentration is determined by subtracting from plasma glucose concentration the contribution of the ingested glucose, which is proportional to the concentration of the meal tracer (e.g., \([1-{^{13}C}]\)glucose) in plasma. Accurate measurement of EGP requires maintenance of the ratio of \([6,6-{2H}_2]\)glucose to the endogenous glucose concentration constant (5, 13, 28). This is more difficult after meal ingestion than it is during a traditional hyperinsulinemic euglycemic clamp, since the infusion rate of both tracer and exogenous glucose is known in the latter but not in the former instance. As previously discussed (28), this ratio can be kept relatively constant by decreasing the infusion rate of the tracer (in this case \([6,6-{2H}_2]\)glucose) in a manner anticipated to mimic the pattern of change of EGP. Clearly, a priori knowledge of the pattern of EGP is required for this approach to succeed. The pattern of suppression of glucose production likely will be influenced by a variety of factors, including age, nutrition, and the presence of a disease (e.g., diabetes mellitus). Therefore, if this information is not already available in the literature, a few pilot studies also may be required to optimize the tracer infusion profile.

Because the triple-tracer approach infused two tracers intravenously in different patterns, it offered the opportunity to assess the validity of 1CM and 2CM in the postprandial setting. In the absence of model errors, the time course of the fractional disappearance rate parameter \(k_{01}\) should be the same for the two tracers, regardless of the format of tracer administra-
tion. As is evident from Fig. 6, the time course of $k_{01}$ was virtually superimposable from 35 min onward when calculated with a 2CM. In contrast, the time course of $k_{01}$ for the two tracers was markedly different when calculated with a 1CM and a fixed glucose distribution volume. The $k_{01}$ for the two tracers only was equal when a time-varying $p$ was used in the 1CM. This unequivocally indicates the superiority of a 2CM compared with a 1CM in the postprandial setting, regardless of the size of the glucose pool assumed for the latter. On the other hand, the comparable rates of meal appearance and EGP with the triple-tracer approach when calculated with either a 1CM or 2CM (see Fig. 4) indicate that the advantage of the latter is minimal when marked changes in the tracer-to-tracer ratio are avoided. Nevertheless, as shown in Fig. 3, it is difficult to maintain either the meal or the endogenous glucose tracer-to-tracee ratio absolutely constant. Therefore, although the error with 1CM likely will be small when used in conjunction with the triple-tracer method, it presumably will be even smaller when the 2CM is used to calculate glucose turnover.

Although the triple-tracer approach is more accurate than the dual-tracer approach, it also is more complex. A key question is whether it is worth the added effort and cost. With the triple-tracer protocol, both intravenous infusions vary in time, thus preventing us from reproducing the conventional dual-tracer approach. This created a worst-case scenario for the dual-tracer approach, since the infusion rate of the intravenous [6,6-2H$_2$]glucose tracer was decreased, whereas it would have been kept constant with the conventional dual-tracer approach. This resulted in more pronounced changes in the relevant tracer-to-tracee ratios than would normally be observed with the conventional dual-tracer approach. As is evident from Fig. 5, the 1CM approach deteriorated when there were marked changes in the tracer-to-tracer ratios. A 2CM performed much better than 1CM, albeit the quality of its results remained inferior to those observed when either 1CM or 2CM was used with the triple-tracer approach. For instance, ISE calculated by the 2CM dual-tracer approach was similar to that estimated with the triple-tracer approach (~15 vs. ~14%); however, individual values were poorly correlated. In addition, EGP could not be reliably calculated with the 2CM dual-tracer approach until ~30 min after the start of the meal. Although these results suggest vulnerability of both 1CM and 2CM dual-tracer approaches to nonsteady state, further experiments are necessary to evaluate the magnitude of the error that would occur when results obtained with the conventional dual-tracer approach are compared with those obtained with the triple-tracer approach. A simulation of the anticipated size of the errors for the 1CM is given in Appendix B. The results, albeit obtained under ideal noise-free conditions, are in keeping with the worst-case scenario.

The present experiment suffers from certain limitations. One limitation is the lack of a gold standard to which the triple-tracer method can be compared. The hepatic catheterization technique is an obvious candidate. However, because tracers also are required for measurement of meal appearance and splanchnic glucose production, the nonsteady state that occurs after food ingestion is problematic for this method as well (17). Only a single meal consisting of scrambled eggs, bacon, and Jell-O was evaluated. Presumably changes in the plasma tracer-to-tracer ratios would have been smaller if the meal had contained less carbohydrate and/or if its composition resulted in slower gastric emptying (e.g., presence of complex carbohydrates). Conversely, the non-steady-state error presumably would have been larger if the meal contained more carbohydrate and/or if glucose alone had been ingested.

Although the triple-tracer approach minimizes error in measurement of EGP and appearance of meal-derived glucose, problems with measurement of glucose $R_d$ persist. Glucose $R_d$ is calculated by subtracting the change in glucose mass from the total rate of glucose appearance. Because the triple-tracer approach minimizes error resulting from tracer nonsteady state, it provides a more accurate measure of total glucose appearance (equal to the sum of endogenous glucose appearance and meal appearance) than does the conventional dual-tracer method. However, both methods calculate the change in glucose mass by multiplying the change in plasma glucose concentration by the $V$ of glucose (i.e., $pV$ in Steele's equations). Therefore, an error in the value (generally assumed) of the volume will introduce error in the calculation of glucose $R_d$.

Finally, values derived from previous studies in normal subjects were used as the exchange rate parameters $k_{21}$ and $k_{12}$ in 2CM (22). Ideally, these parameters should be determined separately in each individual. However, this would require additional sampling for determination of tracer concentrations (e.g., [6,6-2H$_2$]glucose) during the 2–3 h before meal ingestion. The extent to which use of individual vs. population rate parameters improves the performance of 2CM is difficult to predict. It is likely that the improvement would be greater for the 2CM dual- than triple-tracer model. Additional sampling may be feasible in future triple-tracer protocols, since as few as four samples, if placed optimally, are necessary to estimate the two-compartment parameters in a given individual (7).

In summary, the present experiments provide evidence that the rapid and marked changes in the plasma tracer-to-tracer ratios that occur after food ingestion with the conventional dual-tracer method preclude simultaneous measurement of meal appearance and EGP if a 1CM is used to analyze the data. This problem can be minimized by the use of a suitable 2CM and almost completely avoided by use of a triple-tracer approach. Meal appearance measured with the triple-tracer approach is greater and postprandial EGPS smoother than that derived using the dual-tracer approach and a 1CM.

On the other hand, although more accurate, the triple-tracer method also is more complex than the conventional dual-tracer method. Therefore, the triple-tracer method may not be necessary for all experiments. If the purpose of the experiment is to assess meal appearance alone or EGP alone, then only two
tracers are required. In the first instance, if changes in the plasma tracer-to-tracee ratio are to be minimized, the profile of the intravenously infused tracer needs to be varied to mimic the anticipated Ra of the ingested glucose. In the second instance, the profile of the intravenously infused tracer needs to be varied to mimic the anticipated pattern of change of EGP. In contrast, if the purpose of the experiment is to simultaneously assess both meal appearance and EGP, then three tracers are required. The present data also indicate that conclusions reached using the dual-tracer approach in conjunction with the traditional 1CM in ours as well as other investigator’s previous studies needs to be reevaluated and that the triple-tracer technique may provide a useful approach for doing so.

APPENDIX A

1CM

The 1CM of glucose kinetics (22) assumes that Ra and Rd of glucose and glucose tracers take place in the accessible compartment, which has a volume equal to a fraction p of the total glucose distribution volume V. The mass balance equation of [6-3H]glucose is then

\[ pV \times \frac{dG_{3H}}{dt} = F_{3H} - R_{d3H} \]  

(A1)

where \( G_{3H} \) is the concentration of [6-3H]glucose tracer in plasma, \( F_{3H} \) is the rate of [6-3H]glucose infusion, and \( R_{d3H} \) is the rate of [6-3H]glucose tracer disappearance in plasma.

Similarly, for the [1-13C]glucose tracer

\[ pV \times \frac{dG_{13C}}{dt} = R_{a13C} - R_{d13C} \]  

(A2)

where \( G_{13C} \) is the concentration of [1-13C]glucose tracer in plasma, \( R_{a13C} \) and \( R_{d13C} \) are, respectively, the rate of [1-13C]glucose tracer appearance and disappearance in plasma.

Isotopic indistinguishability allows one to link the disappearance rate of the two tracers to their amount in the accessible compartment

\[ \frac{R_{d3H}}{pVG_{3H}} = \frac{R_{d13C}}{pVG_{13C}} \]

(A3)

where \( k_{01} \) is the disappearance rate parameter, which is assumed to vary during the experiment.

By using Eq. A3, Eqs. A1 and A2 can be rewritten as

\[ pV \times \frac{dG_{3H}}{dt} = F_{3H} - k_{01} pVG_{3H} \]  

(A4)

\[ pV \times \frac{dG_{13C}}{dt} = R_{a13C} - k_{01} pVG_{13C} \]  

(A5)

From the [6-3H]glucose mass balance equation (Eq. A4), an expression for \( k_{01} \) (labeled hereafter as \( k_{01,3H} \)) can be derived

\[ k_{01,3H} = - \frac{dG_{3H}/dt}{G_{3H}} + \frac{F_{3H}}{pVG_{3H}} \]  

(A6)

By using Eq. A6 into Eq. A5, one obtains the equation to calculate \( Ra_{13C} \), since either known \( (F_{3H}, pV) \) or measured \( (G_{3H}, G_{13C}) \) variables are needed

\[ Ra_{13C} = \frac{F_{3H}}{G_{3H}/G_{13C}} \times \frac{pVG_{13C}}{G_{3H}/G_{13C}} \frac{dG_{3H}/G_{13C}}{dt} \]  

(A7)

Fig. 10. Plasma tracer-to-tracee ratios during the simulated dual-tracer protocol; the meal glucose appearance and EGP calculated using the 1CM with a pV value of 130, 160, or 230 ml/kg against the “true” rates; the time course of pV that needs to be used to yield rates of meal glucose appearance and EGP that are equal to the true rates.
2CM

The 2CM assumes exchange between the accessible and the remote compartments and irreversible loss from the accessible compartment only.

Mass balance equations of \([6-3H]\) and \([1-13C]\) glucose tracers in the accessible compartment are

\[
V_1 \times \frac{dG_{3H}}{dt} = F_{3H} - (R_{01,3H} + R_{21,3H}) + R_{12,3H} \quad (A8)
\]

\[
V_1 \times \frac{dG_{13C}}{dt} = F_{13C} - (R_{01,13C} + R_{21,13C}) + R_{12,13C} \quad (A9)
\]

where \(R_{01,3H}\) and \(R_{01,13C}\) denote the irreversible flux of the two tracers from the accessible compartment and \(R_{21,3H}\), \(R_{12,3H}\), \(R_{12,13C}\), and \(R_{21,13C}\) are exchange fluxes.

Isotopic indistinguishability provides

\[
\frac{R_{01,3H}}{V_1 G_{3H}} = \frac{R_{01,13C}}{V_1 G_{13C}} = k_{01} = \frac{R_{21,3H}}{V_1 G_{3H}} = k_{21} = \frac{R_{21,13C}}{V_1 G_{13C}} = k_{12} \quad (A10)
\]

The model assumes that parameters \(k_{12}\) and \(k_{21}\) remain constant and that \(k_{01}\) varies during the experiment. By using \(Eq. A10\), Eqs. A8 and A9 can be rewritten as

\[
V_1 \times \frac{dG_{3H}}{dt} = F_{3H} - (k_0 + k_2) V_1 G_{3H} + k_2 Q_{2,3H} \quad (A11)
\]

\[
V_1 \times \frac{dG_{13C}}{dt} = F_{13C} - (k_0 + k_2) V_1 G_{13C} + k_2 Q_{2,13C} \quad (A12)
\]

where \(Q_{2,13C}\) and \(Q_{2,3H}\) are the amounts of \([1-13C]\) glucose and \([6-3H]\) glucose tracer in the peripheral compartment.

From the \([6-3H]\) glucose mass balance equation (\(Eq. A11\)), an expression for \(k_{01}\) (labeled hereafter as \(k_{01,3H}\)) can be derived

\[
k_{01,3H} = -\frac{dG_{3H}/dt}{F_{3H}} + \frac{F_{3H}}{pV G_{3H}} - k_2 + \frac{k_2 Q_{2,3H}}{V_1 G_{3H}} \quad (A13)
\]

By using \(Eq. A13\) into \(Eq. A12\), the following equation for \(R_{a13C}\) is derived

\[
R_{a13C} = \frac{F_{3H}}{G_{3H}/G_{13C}} - \frac{V_1 G_{13C}}{G_{3H}/G_{13C}} \times \frac{d(G_{3H}/G_{13C})}{dt} + k_{12} \left(\frac{Q_{2,3H}}{G_{3H}/G_{13C}} - \frac{Q_{2,13C}}{G_{3H}/G_{13C}}\right) \quad (A14)
\]

Equation A14 expresses \(R_{a13C}\) as a function of known \((F_{3H}, V_1, k_{12}) or \) measured \((G_{3H}, G_{13C})\) variables but also of \(Q_{2,13C}\) and \(Q_{2,3H}\), which are not measured. These last variables were evaluated by solving the mass balance equations of the \([6-3H]\) and \([1-13C]\) glucose tracers in the peripheral compartment

\[
\frac{dQ_{2,3H}}{dt} = -k_{12} Q_{2,3H} + k_{21} V_1 G_{3H} \quad (A15)
\]

\[
\frac{dQ_{2,13C}}{dt} = -k_{12} Q_{2,13C} + k_{21} V_1 G_{13C} \quad (A16)
\]

The procedure outlined in Ref. 17 was followed, which provides equations more suitable for implementation on a spreadsheet.

**APPENDIX B**

To simulate the conventional dual-tracer experiment, a two-compartment model similar to that used to analyze the experimental data (2CM) was adopted. Parameters \(k_{12}\) and \(k_{21}\) were assumed to be constant and equal to 0.07 and 0.05 min\(^{-1}\), respectively, whereas parameter \(k_{01}\) was assumed to vary in time, with a profile equal to the average profile that we found in our 12 subjects with the triple-tracer method and 2CM by using \([6,6-2H_2]\) glucose tracer (Fig. 6A). A value of 130 ml/kg body wt was used for the \(V\) of the accessible compartment.

To simulate the cold (unlabeled) glucose concentration data, model equations were integrated by assuming as initial condition the basal glucose value and as model input the sum of the average \(R_{a\text{meal}}\) plus EGP profiles, estimated with the triple-tracer approach and 2CM (Fig. 4). Similarly, to simulate \([1-13C]\) glucose concentration data, model equations were integrated starting from initial conditions equal to zero and assuming as model input a 5% fraction of average \(R_{a\text{meal}}\). Finally, \([6,6-2H_2]\) glucose concentration data were simulated by assuming a constant infusion of tracer (at a rate equal to 0.48 μmol·min\(^{-1}\)·kg body wt\(^{-1}\)) from 2 h before the meal administration up to the end of the experiment.

The simulations are realistic (Fig. 10): plasma \([6,6-2H_2]\) glucose tracer to the ingested \([1-13C]\) glucose ratio markedly (−7-fold) fell, reaching a nadir ~90 min after meal ingestion. This ratio then slowly returned toward basal values over the 4 h. The plasma ratio of \([6,6-2H_2]\) glucose tracer to total glucose concentration showed a similar pattern. It fell rapidly immediately after meal ingestion (−3-fold), also reaching a nadir at ~90 min followed by a slow increase thereafter (Fig. 3, top right).

On the basis of these data, \(R_{a13C}\) was calculated using the 1CM equation similar to Eq. 16, with \(F_{3H}\) and \(G_{3H}\) in place of \(F_{3H}\) and \(G_{3H}\), respectively. \(R_{a\text{meal}}\) and ISE were calculated as for the triple-tracer method by using Eqs. 11 and 12, respectively. To calculate EGP, \(R_{a}\) was first calculated using an equation similar to Eq. 18, with \(G\) in place of \(G_{\text{end}}\). EGP is then calculated by subtracting \(R_{a\text{meal}}\) and the infusion rate of \([6,6-2H_2]\) glucose from total \(R_{a}\). EGPS is calculated using Eq. 14. To determine the effect of assuming different \(pV\) values in 1CM, calculations were performed with \(pV\) = 130, 160, and 230 ml/kg. Estimates of both \(R_{a\text{meal}}\) and EGP (Fig. 10) show systematic deviations with respect to the true values, which are more consistent when a low \(pV\) value, 130 ml/kg, is used in the calculations.

In Fig. 10, the \(pV\) values required to derive glucose fluxes equal to the true ones are also shown; only the use of a time-varying \(pV\) would theoretically eliminate the errors in glucose flux estimates.

We thank R. Rood, B. Dickie, C. Etter, J. J. Feehan, B. Norby, M. Ott, T. Hammer, and L. Wahlstrom for technical assistance and assistance in recruiting the subjects. M. Davis for assistance in the preparation of the manuscript, and the staff of the Mayo General Clinical Research Center for assistance in performing the studies. We also thank other members of the program project team, including Drs. S. Nair, M. Jensen, and S. Khosla, for thoughtful suggestions.

This study was supported by the U.S. Public Health Service (AG-14383 and RR-00585), a Novo Norskisk research infrastructure grant, the Ministero Universita Ricerca Scientifica Tecnica (Murst), Italy, and the Mayo Foundation. Dr. R. Basu was supported by an American Diabetes Association mentor-based fellowship.

**REFERENCES**


