Simultaneous time-varying systemic appearance of oral and hepatic glucose in adults monitored with stable isotopes

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Livesey, G., P. D. G. Wilson, J. R. Dainty, J. C. Brown, R. M. Faulks, M. A. Roe, T. A. Newman, J. Eagles, F. A. Mellon, and R. H. Greenwood. Simultaneous time-varying systemic appearance of oral and hepatic glucose in adults monitored with stable isotopes. Am. J. Physiol. 275 (Endocrinol. Metab. 38): E717–E728, 1998.—The rates (and extent) of appearance of glucose in arterialized plasma from an oral glucose load and from liver (RaO, RaH) can be estimated in humans using radioisotopes, but estimates vary among laboratories. We investigated the use of stable isotopes and undertook 22 primed intravenous infusions of D-[6,6-2H2]glucose with an oral load including D-[13C6]glucose in healthy humans. The effective glucose pool volume (Va0) had a lower limit of 230 ml/kg body weight (cf. 130 ml/kg commonly assumed). This Va0 in Steele’s one-compartment model of glucose kinetics gave a systemic appearance from a 50-g oral glucose load per 70 kg body weight of 96 ± 3% of that ingested, which compared with a theoretical value of ~95%. Mari’s two-compartment model gave 100 ± 3%. The two models gave practically identical RaO and RaH at each point in time and a plateau in the cumulative RaO when absorption was complete. Less than 3% of [13C3]glucose was recycled to [12C3]glucose, suggesting that recycling errors were practically negligible in this study. Causes of variation among laboratories are identified. We conclude that stable isotopes provide a reliable and safe alternative to radioactive isotopes in these studies.

DUAL ISOTOPE MONITORING of time-varying rates of entry into the systemic circulation of glucose from the gut and the liver in humans generally involves a primed continuous intravenous infusion with labeled glucose, oral loading with differently labeled glucose molecules, and simultaneous intermittent sampling of blood plasma for the analysis of isotopic species. In humans, this technique is growing in popularity but has been dependent on radioisotope labels (7–9, 11, 12, 16, 17, 20, 21, 23, 26, 28, 29), although not exclusively (6). However, stable isotopic labels offer an alternative, albeit expensive, means of safely and ethically meeting the need for studies of glucose metabolism. Abnormalities in glucose metabolism are central to current hypotheses on obesity and diabetes and risk factors linked with coronary artery disease (31, 36). The abnormalities appear early in the disease process (24), and the disease conditions are increasingly prevalent (19, 34). We have, therefore, determined whether the use of the mass isotoomers D-[6,6-2H2]glucose and D-[13C6]glucose in place of their radioactive 3H and 14C counterparts in the dual isotope method presents unexpected obstacles when used with oral glucose loading. On the basis of our stable isotope data and observations that we summarize from the literature (for references see Table 1), we identify possible errors in published results. Such errors were substantial, explaining large differences (up to 20% of expected results) in reported estimates of oral glucose appearance in the systemic circulation, and much larger differences (up to ≥300% of expected results) in estimates for splanchnic retention of glucose during orosystemic passage.

In assessing the usefulness of stable isotopes for this purpose, it is prudent to examine the performance of models chosen to make rate estimates. Previous dual radioisotope work with oral loading has been conducted using one- and two-compartment models (see Fig. 1 and Refs. 5, 21, 30), but substantially different model- or parameter-dependent rates of glucose entry from the liver and gut have been estimated. A theoretical analysis indicates that different rates can arise from either volume or structure errors or both (5). Because there has been no experimental assessment of the models by use of dual stable isotopes, nor any estimation of the effective glucose distribution volume in this situation, we examine both in the present study.

One model we chose to assess was a simple one-compartment model developed by Steele, although this has been criticized as not representing the underlying physiology of glucose kinetics (5, 33). Steele’s model has often, and recently, been used with radioisotopes in the dual tracer method (9, 10, 12, 13, 20, 22, 27), and it has been employed recently in a dual tracer study involving stable isotopes and oral loading with glucose (6). Furthermore, we have used Steele’s one-compartment model in dual isotope studies with 13C-labeled starch and D-[6,6-2H2]glucose, reported in early communications (14, 15). It is appropriate, therefore, to establish whether or not Steele’s model can yield realistic quantitative results when either radioactive or stable isotopes...
are used to monitor the perturbation of metabolism resulting from an oral load.

A weakness in Steele’s model to which he drew attention (33) has now been demonstrated in situations involving rapid or step changes in intravenous glucose loading, such as the euglycemic clamp and minimal models applied to data from intravenous glucose tolerance tests (2, 13, 27). In these situations, glucose appearance in plasma is underestimated by Steele’s model when compared with the known unlabeled glucose infusion rates. The weakness in the model results in an underestimation of hepatic glucose production to an extent that unacceptable negative rates are calculated (5, 13). This problem is largely overcome by the use of two- or higher-order models (6, 8) and by minimizing changes in the tracer-to-tracee ratio (2, 13, 27, 33). Interestingly, by contrast, in dual radioisotope studies involving oral loading, application of Steele’s model can result in higher (not lower) estimates of hepatic glucose production compared with estimates from two- or higher-order models (5, 7), but the extent to which this is, in practice, due to the model or to the distribution volume is unclear. The present study determines whether a one-compared with a two-compartment model necessarily yields substantially different systemic appearance rates of glucose from liver and gut during application of the dual isotope method when mass isotopes are used. In the present study, we use oral glucose loads that yield near to habitual postprandial plasma glucose concentrations.

Two-compartment models offer more complex and arguably more dynamically realistic approaches than Steele’s model when rates of glucose entry into the circulation change rapidly. Of such models that have been proposed, only those of Radziuk et al. (30) and Mari et al. (18) have been extensively validated; these use radioisotopes under several experimental conditions, including, importantly, the dual radioisotope method. Both models give plausible results, so that a decision at present to choose one in preference to the other appears to be arbitrary. Mari et al. developed their model from the basis of work by Radziuk et al. and, although structurally very similar, can be solved in a simpler and more intuitive manner using a spreadsheet called SMART (17). The only precondition for this approach is a steady-state period before the nonsteady perturbation with oral glucose. The spreadsheet makes Mari’s model an accessible analysis tool that we chose to use for comparison with Steele’s one-compartment model. A feature of the software is the simultaneous estimation of error bounds, which is essential to the objective comparison of the two models.

Finally, no authors appear to have determined an effective glucose distribution volume in humans for application specifically with Steele’s or equivalent one-compartment models when dual isotopes and oral loading are applied. Not only is this distribution volume difficult to measure in a way that is suitable for application with experimental data, but the effective volume depends on the time scale over which it is measured. This is because of the longer time scale for mixing at the “extremities” of the pool. We present a stable theoretical approach to the derivation of the distribution volume, together with parameter estimates derived from the primed-constant infusion of $\delta$-[6,6-$^3$H$_2$]glucose. As will be discussed, the determination and choice of correct distribution volume are important for the estimation of the least erroneous rates of glucose entry into the systemic circulation.

**METHODS**

**Participants**

Thirteen “healthy” volunteers with no family history of diabetes mellitus were recruited. Eight were female and five were male, and they were 19–59 (39 ± 14 SE) yr old, weighed 61–104 (79 ± 11) kg, and had body mass index of 22–37 (28 ± 4) kg/m$^2$. They ate their habitual diets for ≥6 wk before investigation, were weight stable (± 2 kg), and were nonmedicated. Investigations were repeated in nine of the volunteers, which gave a total of 22 investigations. Informed written consent was provided by all participants, and the study was approved by both the Norfolk and Norwich Health District Ethics Committee and the Institute of Food Research Human Research Ethics Committee.

**Experimental Design**

Volunteers were fasted for 12 h overnight before being seated for up to 10 h in a recliner chair (Parker Knoll, Chipping-Norton, UK), with ambulation limited to two toilet visits. An antecubital vein and a subsequently heated dorsal hand vein were cannulated (18-gauge Teflon) and kept patent with physiological saline. Pyrogen-free $\delta$-[6,6-$^3$H$_2$]glucose (99 mol% enrichment, C/D/N Isotopes, Croydon, UK) was administered as a primed (500 mg in a 10 ml aqueous solution)-continuous (6 mg/min as a 68.5 g/l aqueous solution) infusion into an antecubital vein, starting at –0900, denoted time 0 in RESULTS. After 2 h, we administered peroral $\delta$-glucose (50 g in 200 ml water, each per 70 kg body weight) that was labeled with $\delta$-[13C$_6$]glucose (99 mol% enrichment, C/D/N Isotopes, K&K Greeff, Croydon, UK) as a COBAS MIRA centrifugal analyzer (Welwyn Garden City, UK). The coefficient of variation on replicate analyses was 0.014 for a normal basal concentration of 5.3 mmol/l glucose in plasma, and the percentage recovery of a doubling spike was 100.5 ± 0.8 SE. The coefficient of variation in the quality control for plasma glucose (mean 4.5 mmol/l) analyzed on repeated occasions was 0.016. Glucose isotopomer ratios were determined on butyl boronic acid acetate derivatives using gas chromatography-electron-impact mass spectrometry (25). Protein in plasma (200 µl = 1 vol) was precipitated with 2 volumes of 2-propanol onto a bed mixed resin, Merck) for 15 min. After filtration (Gelman Acrodisc LC13) and washing through with 5 volumes of 2-propanol, the extract was dried at 50°C under nitrogen gas...
with a sample concentrator (Techne, Cambridge, UK) and then freeze-dried after it was first dissolved in 2 volumes of water. After derivatization (25), the dry residue was taken into trimethylpentane containing 1% acetic anhydride. A sample was injected into a Hewlett-Packard 5890 Series II gas chromatograph containing a BD-SMS column (Fisons, Loughborough, UK) under conditions previously described (25). Ions generated by electron impact were selectively monitored in a Trio 1-S mass spectrometer (Finnigan MASSLAB, Manchester, UK) at mass-to-charge (m/z) 297, 299, 300, and 303 for molecular enrichments M, M+2, M+3, and M+6, respectively, which correspond to natural-[6,6-2H2], [13C2], [13C3], and [13C6]glucose molecules, respectively. Plasma isotope enrichments were determined from linear standard curves of 5–40 mg of D-[6,6-2H2]glucose and 0.736 mg of D-[13C6]glucose/g total glucose had coefficients of variation of 0.048 and 0.039, respectively, when analyzed on repeated occasions. No such standards were available for the [13C3]glucose produced by the recycling of 13C back into glucose. The occurrence of [13C5]glucose in plasma was calculated from the relative abundance (areas) of m/z ions at 303 (M+6), 300 (M+5), 299 (M+2), and 297 (M). To do this, the theoretical mass spectra of each derivative were calculated from a consideration of the derived elemental and isotopic composition of each glucose isotopomer and the natural abundance of the elements added during derivatization. The spectrum of the mixture of the derivatized glucose isotopomers was a linear combination of the spectra of each of the components. In this way, four equations (one for each of the m/z ions) were set up with four unknowns (the mole fraction of glucose derived from each of the isotopomers). These equations were solved to give the mole fraction of total glucose present as the (M+3) isotopomer.

Rate of Appearance Calculations

The rates at which glucose appeared in arterialized plasma from the exogenous (oral) and endogenous (hepatic) sources (RaO and RaH) were estimated by both Mari’s two-compartment and Steele’s one-compartment models (Fig. 1) (16, 33). A term that estimates glucose appearance rate Ra(t) in the steady state (Eq. 1) was present in each model (Eqs. 2 and 3); RaS is the known rate of tracer infusion. The second term in each model was essentially identical, differing only in the chosen volume of the sampled compartment, abbreviated to Vs in Steele’s model and V1 in Mari’s model. The two-compartment model included a third term Ra(t) (Eq. 3) to describe a rate of (re)appearance of glucose in the sampled compartment (V1) from the unobservable compartment. Computation of Ra(t) used a second pool volume, V2, and was complex, and so for brevity we refer to Mari (16). Mean parameter values Vs (146 ml/kg), V2 (84 ml/kg), k21 = k10 + k12 (0.067 min−1), and sum of volumes V1 + V2 = 230 ml/kg were as derived previously (18). Steele’s volume (Vs) was estimated by using data from the above protocol (see below).

Measurable plasma glucose concentrations were [6,6-2H2]+[13C5]glucose, [13C2]glucose, [13C3]glucose, [13C6]glucose, and total glucose (labeled and unlabeled). To obtain [6,6-2H2]glucose, the contribution to the M + 2 peak from [13C2]glucose was assumed to be negligible (e.g., the present computations of RaO and RaH); otherwise, the contribution was assumed to be small and equal in size to the M + 3 peak (i.e., M + 2 ≈ M + 3; Ref. 3). The latter assumption permitted rate data to be recalculated and errors from assumed negligible recycling to be estimated. Identical behavior of labeled and unlabeled glucose molecules was assumed, so that the concentrations in plasma of glucose derived from both the liver and gut could be calculated.

Endogenous or hepatic glucose (i.e., total glucose less glucose traced with D-[13C6]glucose and D-[6,6-2H2]glucose) and oral glucose (traced with D-[13C6]glucose) were the tracers in the computational models, and intravenous glucose ([6,6-2H2]glucose) was the tracee (Eqs. 2 and 3). Thus endogenous glucose included recycled glucose. Recycling of [13C5]glucose was also estimated by using the latter as tracer in place of D-[13C6]glucose.

By substituting the appropriate trace concentration into Eqs. 2 and 3, RaO and RaH were calculated using SMART (17), a program developed for the analysis of the rate of appearance of substances in the nonsteady state. The program smoothed both tracer and tracee concentrations and interpolated at time intervals of 5 min to aid integration.

Steady state

\[ R_a(t) = \frac{R_a^*}{a(t)} \] (1)

Steele’s equation

\[ R_a(t) = \frac{R_a^*}{a(t)} - V_s \frac{C(t) a(t)}{a(t)} \] (2)

where \( a(t) \) was the tracer-to-tracee concentration ratio, i.e., \( C(t)/C(t) \), and \( a(t) \) was its derivative with respect to time.

Mari’s equation

\[ R_a(t) = \frac{R_a^*}{a(t)} - V_l \frac{C(t) a(t)}{a(t)} + R_2(t) \] (3)
GLUCOSE KINETICS IN HUMANS AFTER AN ORAL LOAD

The function $R_2(t)$ was complex. It gave an estimate of the contribution to $R_a$ of the second compartment and was dependent on the values of $k_{0_2}$, $k_{12}$, $V_2$, $C(t)$ and $C'(t)$ (16).

Statistics, Error Analysis, and Model Superiority

There were 13 subjects, and of these, 9 subjects repeated the investigation. Calculations using the one- and two-compartment models were made for each of the 22 investigations, and the results were summarized as mean values. Repeated-measures analysis of variance on sampled data showed that between-subject variation was frequently insignificant, particularly for rate data, and so each investigation was treated as a separate observation, with the associated SE of the mean being determined separately at each time point. Significance of difference was tested using the least significant difference test. $R_{aO}$ and $R_{aH}$ had means that were practically identical to their medians, and normal probability plots indicated that distributions were approximately Gaussian.

The calculated rates of appearance were subject to error, because models of necessity approximate the true system. A thorough analysis of Steele's model (5) has identified volume because models of necessity approximate the true system. A contribution to $R_a$ of the second compartment and was complex. It gave an estimate of the pool size and the concentration of tracer immediately after injection of the bolus. In practice, however, the mixing of the tracer is not rapid enough to allow this estimate. We can, nevertheless, learn about the size of the glucose pool by considering the time course of the tracer concentration. Using the assumption that the pool size remains at a steady state during priming, i.e., that the rate of glucose disappearance equals the sum of the endogenous appearance plus the tracer infusion rate, we may write a mass balance for the tracer concentration, describing the approach to a steady-state enrichment as follows:

$$\frac{dx}{dt} = R_a^* - (R_a + R_b^*) x$$

(8)

This equation may be integrated to give the time course of tracer

$$x(t) = \frac{\alpha - (\alpha - \beta x_0) e^{-\beta t}}{\beta}$$

(9)

where

$$\alpha = \frac{R_a^*}{P}, \quad \beta = \frac{R_a + R_b^*}{P}$$

(10)

Eq. 9 describes an exponential rise or fall to a steady-state enrichment ($x_{ss}$) given by

$$x_{ss} = \frac{R_a^*}{R_a^* + R_b^*}$$

(11)

Whether $x$ rises or falls to this limit depends on the size of the initial bolus with respect to the pool size and the two rates of appearance. If the tracer concentration rises to the limit, i.e., $(dx/dt) > 0$, it can be shown (by use of Eqs. 8 and 10) that

$$P > B \left[ \frac{R_a^* + R_b^*}{R_a^*} - 1 \right]$$

(12)

If the tracer concentration falls to the limit, then the inequality is reversed. More generally, we may define a function, $z(t)$

$$z(t) = B \left[ \frac{1}{x(t)} - 1 \right]$$

(13)

At $t = 0$, Eq. 13 reduces to Eq. 7, giving $z_0$ equal to the pool size as a function of the (unmeasurable in practice) initial tracer concentration. As $t \rightarrow \infty$, Eq. 13 reduces to Eq. 12, giving a lower limit on the pool size. Equation 9 already demonstrates that $x(t)$ changes monotonically and with a constant direction of curvature with time, and so from Eq. 13 we know that $z(t)$ also does. Hence, we can extrapolate a plot of $z(t)$ back to $t = 0$ to obtain an estimate of the pool size, $P$. A linear extrapolation will always give a lower bound for $P$ when the tracer rises to a limit, and an upper bound when the tracer falls. Taking values of $x(t)$ and linear extrapolation to $z_0$ was a more stable procedure than taking the values of $x$ and curve fitting to $x_0$: this was because the linear approach, which produces a bound on the pool size, has one less parameter to estimate.

Substituting the time course for $x(t)$ from Eq. 9 into Eq. 13 allows us to make an estimate of how close to the real value our estimate will be, given a knowledge of true pool size and hepatic output. For the experiment carried out in this study, the worst case (extrapolating a tangent to the curve at $t = 120$ min back to $t = 0$) would give an underestimate of 24%. A better estimate of the error is a line passing through $z(t)$ at $t = 30$ and $t = 120$ min, which indicates an underestimate of
3.8%. \( V_S \) was estimated at each investigation, but a pooled estimate was used every time Steele’s model was applied, because individual estimates were based on only a few sampling intervals.

**RESULTS**

**Mean Arterialized Plasma Glucose Concentrations**

The concentration of the \( M+2 \) glucose, which is the sum of \([6,6-\text{D}_2]\) and \([^{13}\text{C}_2]\)glucose, increased (Fig. 2) to approach a steady state close to \(0.18 \pm 0.01 \text{ mmol/l} \) during the 2 h immediately after the start of the primed-continuous infusion and before the start of the nonsteady perturbation with oral glucose (50 g glucose/70 kg body weight). The perturbation decreased the tracer concentration to a nadir at 3 h after ingestion, \(-30\% \) below that in the near steady state. By 5 h after ingestion, the near-steady-state concentration had been reached again. A similar pattern of observations occurred in all volunteers, which gave rise to the narrow error values. The concentration of \( M+3 \) glucose (label recycled into \([^{13}\text{C}_3]\)glucose) began to rise immediately after the oral load of \( \delta-[^{13}\text{C}_6] \)glucose at 120 min (Fig. 2). By the end of the infusion, the \( M+3 \) peak had reached \(2.8 \pm 0.3\% \) of the \( M+2 \) peak, which was a slight fall from a maximum mean percentage of \(2.9 \pm 0.9\% \) at 330 min. A reasonable assumption that recycling produced \([^{13}\text{C}_2]\)glucose in quantities similar to \([^{13}\text{C}_3]\)glucose (3) allows that, on average, \( >97\% \) of the \( M+2 \) glucose was the tracer \( \delta-[6,6-\text{D}_2] \)glucose.

After its ingestion, \( \delta-[^{13}\text{C}_3] \)glucose soon appeared in the arterialized plasma (Fig. 3) and peaked within \(1.5\ h \), at a concentration of \(7.0 \pm 0.3 \text{ mmol/l} \), before returning \(80\% \) of the way toward zero again at the close of the investigation.

The concentration of glucose that originated from the liver decreased soon after ingestion of the glucose drink and reached a nadir of \(1.1 \pm 0.1 \text{ mmol/l} \) at 3 \(h \) after ingestion. Between 3.5 and 5 \(h \) after ingestion, the decreasing concentration of glucose derived from the oral dose was matched by an increasing concentration of glucose from the liver, a process that was \(-50\% \) completed 5 \(h \) after ingestion of the glucose drink.

The total glucose concentration in the arterialized plasma was (by definition) the sum of concentration of glucose from the liver, oral load, and infusion. From a basal concentration of \(2.2 \pm 0.1 \text{ mmol/l} \), it peaked at \(8.8 \pm 0.4 \text{ mmol/l} \) at \(-1\ h \) after ingestion and then returned to a new “plateau” of \(3.8 \pm 0.2 \text{ mmol/l} \) between 3.5 and 5 \(h \) after ingestion.

**Estimation of Steele’s Pool Volume**

At each investigation, pool volume estimates were computed (Eq. 13, \(z_0 \) when \(t = 0 \)) from the primed-continuous infusion rate and the rise in \( \delta-[6,6-\text{D}_2] \)glucose concentration before 2 \(h \) (Fig. 2), when contribution to the \( M+2 \) peak from \([^{13}\text{C}_3]\)glucose was absent. The estimate increased with increasing body weight (Fig. 4), and linear regression indicated a mean value of \(0.22 \pm 0.01 \text{ l/kg when zero pool volume was forced at zero body weight. The results from the five male volunteers were not distinguishable from those for the eight female volunteers (P > 0.1).} \)

**Mean Rate of Systemic Appearance of Hepatic Glucose**

The mean basal steady-state \( R_{WH} \) was \(2.2 \pm 0.1 \text{ mg·min}^{-1}·\text{kg body weight}^{-1} \), whether it was obtained with equations of the one- or the two-compartment model. Oral glucose acutely suppressed \( R_{WH} \) by \(-90\% \) to a nadir at \(1.1 \) h after glucose ingestion at \(0.2 \pm 0.2 \text{ mg·min}^{-1}·\text{kg}^{-1} \) (Fig. 5). Thereafter, \( R_{WH} \) increased, returning to \(75\% \) of the basal value by \(5 \) h after the oral glucose.
Choosing an upwardly rounded value for the lower bound of the glucose pool volume (230 ml/kg; cf. Fig. 4) resulted in $R_{ah}$ estimates by the one- and the two-compartment models that were nearly identical throughout the non-steady-state period (Fig. 5). Estimates using the one-compartment model were well within the error bounds for the two-compartment model (Fig. 5), and at no time did the inequality term $E(t)/R(t)$ (Eq. 6) fall to a significant value (<0.5), but frequently reached nearly 100. Thus the two-compartment model was far from superior for the present application.

Mean Rate of Systemic Appearance of Oral Glucose

As with $R_{ah}$, values of $R_{ao}$ were similar in the non-steady-state period when estimated by the one- and the two-compartment models (Fig. 6). In both models, $R_{ao}$ accelerated to a peak of 6.2 ± 0.2 mg·min⁻¹·kg body weight⁻¹ at ~50 min after glucose ingestion, peaking only slightly ahead in the higher-order model. The rate later decelerated, over a longer time period, to near zero by 3 h after glucose ingestion. Rates estimated using the one-compartment model were, again, well within the error bounds for the two-compartment model (Fig. 6), and at no time did the inequality term $E(t)/R(t)$ (Eq. 6) fall to a significant value (<0.5). Thus the two-compartment model was again not significantly superior for this application.

Cumulative Appearance of Oral Glucose

The rates of oral glucose appearance in the peripheral circulation (Fig. 6) gave a poor indication of the extent to which the glucose load was absorbed. Cumulative absorption and peripheral appearance were calculated by integration of the $R_{ao}$ data and expressed as a fraction of the glucose ingested. Such estimates of fractional systemic appearance indicated 1.00 ± 0.03 of the oral dose reaching the periphery when the two-compartment model was used, and 0.96 ± 0.03 when the one-compartment model was used (Fig. 7). In both models, the systemic glucose appearance reached a plateau ~3 h after oral glucose. SE values were reasonably small.

The reappearance of label from oral $D-[^{13}C_6]$glucose as $M^{13}$ glucose (i.e., $[^{13}C_3]$glucose) was calculated using the one-compartment model (Fig. 7). After oral glucose, the systemic appearance of $M^{13}$ glucose rose steadily and continued to rise even after the systemic appearance of $[^{13}C_6]$glucose reached plateau. When this plateau was reached, by 300 min, just 1.6 ± 0.3% of the label from oral $[^{13}C_6]$glucose had reappeared in $M^{13}$ glucose, and by the end of the experimental period, at 420 min, it had reached just 2.9 ± 0.5%.
Assessment of Errors

We consider first the errors that arise from an inappropriate choice of Steele’s volume $V_S$, and then those that arise from assuming that measurements of M+2 glucose are solely due to the tracer $\text{D-[6,6-2H}_2\text{]}$glucose.

Values of $V_S$ applied in studies with oral glucose range from 95 to 230 ml/kg body weight (Table 1). Departure from the $V_S$ applied at present (230 ml/kg) by just 10 ml/kg resulted in values for $R_\text{ah}$, $R_\text{ao}$, and cumulative systemic appearance of oral glucose that varied time dependently, and maximally by between 1.5 and 3%. Expressing these percentage values as fractions (0.015 and 0.030) gave the time-dependent fractional sensitivities of these rates to $V_S$ (fraction per 10 ml/kg body wt; bold curve, appearance from the gut according to Mari’s 2-compartment model; lowest curve, appearance of M+3 glucose (i.e., [13C]glucose); vertical bars, corresponding SE.

The contribution to the M+2 peak from [13C] label recycled into [13C]glucose was not measurable in the present circumstances because of the infusion of $\text{D-[6,6-2H}_2\text{]}$glucose. Such a contribution would have caused systemic appearance of glucose to be underestimated because of overestimation of tracer $\text{D-[6,6-2H}_2\text{]}$glucose present in plasma. However, it can be assumed that the concentration of [13C]glucose in plasma is approximately similar to the concentration of [13C]glucose; this is because the M+2 and M+3 sugars are generated from [13C]glucose in approximately similar quantities in humans (and other species) (3). Making this assumption, we calculated the extent to which systemic appearance of oral glucose would be underestimated (Fig. 9). By the time the systemic glucose appearance...
Table 1. Models, isotopes, assumptions, and parameter estimates for systemic glucose appearance before and after an oral glucose load in adult volunteers

<table>
<thead>
<tr>
<th>Model Structure</th>
<th>Isotopes</th>
<th>Basal Hepatic Glucose Production, mg·min⁻¹·kg⁻¹</th>
<th>Glucose Dose (and Body Weight, kg)</th>
<th>Peak RaO₂, mg·min⁻¹·kg⁻¹</th>
<th>Extent of Appearance in Plasma, %</th>
<th>Time, h (Frequency)</th>
<th>No. of Subjects</th>
<th>Reference (No.)</th>
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<tr>
<td>2a</td>
<td>3-3H/1-3C</td>
<td>Variable</td>
<td>d 50 g</td>
<td>d 93</td>
<td>4(F)</td>
<td>4 N</td>
<td>Radziuk et al. 1978 (29)</td>
<td></td>
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<tr>
<td>2a</td>
<td>3-3H/1-3C</td>
<td>Variable</td>
<td>d 93 g ( –65)</td>
<td>d 92 ± 2</td>
<td>4.8(F)</td>
<td>5 N</td>
<td>Radziuk 1979 (28)</td>
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<tr>
<td>2a</td>
<td>3-3H/1-3C</td>
<td>65 135</td>
<td>2.37 ± 0.11 1 g/kg (–60)</td>
<td>5.5 ± 1</td>
<td>72 ± 7</td>
<td>3.5(I)</td>
<td>Piloti et al. 1981 (26)</td>
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<tr>
<td>1</td>
<td>2-3H/6,6-2H</td>
<td>130</td>
<td>2.4 ± 0.3 83 g (69)</td>
<td>3.5 ± 0.5</td>
<td>92</td>
<td>8.0(F)</td>
<td>Pehling et al. 1984 (23)</td>
<td></td>
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<tr>
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<td>2-3H/6,6-2H</td>
<td>130</td>
<td>1.8 ± 0.3 87 g (73)</td>
<td>4.9 ± 0.8</td>
<td>93</td>
<td>8.0(F)</td>
<td>Pehling et al. 1984 (23)</td>
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<tr>
<td>1</td>
<td>2-3H/6,6-2H</td>
<td>130</td>
<td>4.5 ± 0.7 87 g (73)</td>
<td>3.8 ± 0.6</td>
<td>93</td>
<td>8.0(F)</td>
<td>Pehling et al. 1984 (23)</td>
<td></td>
</tr>
<tr>
<td>2a</td>
<td>3-3H/1-3C</td>
<td>Variable</td>
<td>1.92 ± 1.02 1 g/kg (66)</td>
<td>6.3 ± 0.3</td>
<td>76 ± 9</td>
<td>3.5(F)</td>
<td>6 5 11 N</td>
<td>Ferrannini et al. 1985 (7)</td>
</tr>
<tr>
<td>2a</td>
<td>2-3H/6-3H</td>
<td>130</td>
<td>3.04 ± 0.24 50 g (84)</td>
<td>3.0 ± 0.4</td>
<td>92</td>
<td>5.0(F)</td>
<td>9 4 13 N</td>
<td>Firth et al. 1986 (9)</td>
</tr>
<tr>
<td>2a</td>
<td>2-3H/6-3H</td>
<td>130</td>
<td>1.84 ± 0.10 50 g (89)</td>
<td>2.1 ± 0.4</td>
<td>104</td>
<td>5.0(F)</td>
<td>9 4 13 N</td>
<td>Firth et al. 1986 (9)</td>
</tr>
<tr>
<td>2a</td>
<td>2-3H/6-3H</td>
<td>130</td>
<td>2.00 ± 0.01 50 g (84)</td>
<td>3.5 ± 0.4</td>
<td>92</td>
<td>5.0(F)</td>
<td>6 1 7 N</td>
<td>Firth et al. 1986 (9)</td>
</tr>
<tr>
<td>2a</td>
<td>2-3H/6-3H</td>
<td>130</td>
<td>1.96 ± 0.04 1 g/kg (74)</td>
<td>4.3 ± 1</td>
<td>71 ± 5</td>
<td>5.0(I)</td>
<td>4 6 10 N</td>
<td>Mitrakou et al. 1990 (21)</td>
</tr>
<tr>
<td>2a</td>
<td>2-3H/6-3H</td>
<td>130</td>
<td>2.26 ± 0.11 1 g/kg (79)</td>
<td>4.9 ± 0.4</td>
<td>72 ± 1</td>
<td>5.0(I)</td>
<td>4 6 10 N</td>
<td>Mitrakou et al. 1990 (21)</td>
</tr>
<tr>
<td>2a</td>
<td>2-3H/6-3H</td>
<td>130</td>
<td>2.04 ± 0.08 1 g/kg (84)</td>
<td>4.3 ± 0.5</td>
<td>77 ± 1</td>
<td>5.0(I)</td>
<td>4 6 10 N</td>
<td>Mitrakou et al. 1990 (21)</td>
</tr>
<tr>
<td>2a</td>
<td>2-3H/6-3H</td>
<td>130</td>
<td>2.11 ± 0.11 1 g/kg (74)</td>
<td>4.9 ± 0.4</td>
<td>73 ± 2</td>
<td>5.0(I)</td>
<td>4 5 9 N</td>
<td>Mitrakou et al. 1992 (20)</td>
</tr>
<tr>
<td>2a</td>
<td>2-3H/6-3H</td>
<td>130</td>
<td>2.11 ± 0.07 1 g/kg (73)</td>
<td>5.2 ± 0.4</td>
<td>72 ± 2</td>
<td>5.0(I)</td>
<td>1 7 8 GI</td>
<td>Mitrakou et al. 1992 (20)</td>
</tr>
<tr>
<td>2a</td>
<td>2-3H/6-3H</td>
<td>130</td>
<td>1.80 ± 0.09 1 g/kg (84)</td>
<td>4.5 ± 0.4</td>
<td>73 ± 2</td>
<td>5.0(I)</td>
<td>4 3 7 O</td>
<td>Mitrakou et al. 1992 (20)</td>
</tr>
<tr>
<td>2a</td>
<td>2-3H/6-3H</td>
<td>130</td>
<td>1.95 ± 0.08 1 g/kg (84)</td>
<td>4.9 ± 0.4</td>
<td>73 ± 1</td>
<td>5.0(I)</td>
<td>5 2 7 O + GI</td>
<td>Mitrakou et al. 1992 (20)</td>
</tr>
<tr>
<td>1</td>
<td>2-3H/6-3H</td>
<td>95</td>
<td>2.1 ± 0.2 75 g (73)</td>
<td>4.7 ± 0.3</td>
<td>72 ± 3</td>
<td>4.0(F)</td>
<td>8 N</td>
<td>Kruszynska et al. 1993 (12)</td>
</tr>
<tr>
<td>1</td>
<td>2-3H/6-3H</td>
<td>95</td>
<td>2.4 ± 0.1 75 g (74)</td>
<td>5.8 ± 0.3</td>
<td>72 ± 3</td>
<td>4.0(F)</td>
<td>8 N</td>
<td>Kruszynska et al. 1993 (12)</td>
</tr>
<tr>
<td>1</td>
<td>2-3H/6-3H</td>
<td>130</td>
<td>1.63 ± 0.09 65 g (85)</td>
<td>3.8 ± 0.2</td>
<td>87</td>
<td>5.0(I)</td>
<td>4 5 9 N</td>
<td>Féry and Balasse 1994 (8)</td>
</tr>
<tr>
<td>1</td>
<td>2-3H/6-3H</td>
<td>p = 0.165</td>
<td>0.85 ± 0.06 65 g (83)</td>
<td>3.0 ± 0.2</td>
<td>85</td>
<td>5.0(F)</td>
<td>3 5 8 N</td>
<td>Féry and Balasse 1994 (8)</td>
</tr>
</tbody>
</table>

One-compartmental models are sometimes referred to as Steele's model. Authors using 1-compartment models often indicate modifications to total pool volume (V₁) and pool fraction (p); tabulated value of V₁ ether equals pV₁, or in the present study is estimated by noncompartmental analysis. V₂, Steele's volume; V₃, volume of compartment 1; V₄, volume of compartment 2; RaO₂, rate of glucose appearance from oral glucose load; F, frequent sampling in critical period; I, infrequent sampling in critical period; N, normal volunteers (overnight fasted); NIDD, non-insulin-dependent diabetes mellitus (c, controlled or u, uncontrolled with insulin, both overnight fasted); NIDD, non-insulin-dependent diabetes mellitus overnight fasted, or 4dfast, fasted for 4 days; O, obese fasted; GI, glucose intolerant and fasted; C, cirrhotic fasted patients; F, M, and T subjects, females, males, and total. Sometimes referred to as Radziuk's or Norwich's model, sometimes referred to as Mari's model. Radziuk made no a priori assumptions about sizes of the 2 pool volumes but determined them from primed-continuous infusion rates and concentrations in plasma of isotopes during first 2 h. Body weights were not reported. *Dose 1 g/kg up to a maximum of 75 g.

had approached plateau in Fig. 7, at 300 min, the appearance was underestimated by just 1.7 ± 0.3%, and by the end of the experiment, at 420 min, by just 2.2 ± 0.2%. This suggests that recycling of glucose leads to practically negligible errors, even though the error estimates are statistically significant.

DISCUSSION

Stable isotopes have been used increasingly for monitoring both steady-state and time-varying rates of glucose turnover in humans during intravenous glucose loading, for example in euglycemic clamps and labeled intravenous glucose tolerance tests (for examples and references see Refs. 2, 27). However, there is a relative absence of data when the glucose is administered orally, a condition which is particularly relevant to meal-induced perturbation of metabolism. The last situation requires dual isotope methodology, and various combinations of isotopes have been used (Table 1), but they have been almost exclusively radioactive isotopes. In the present study, we investigate [6,6-²H₂]glucose and [13C₆]glucose as stable isotope labels to estimate the systemic appearance rates of both oral and hepatic glucose (RaO₂ and RaH₂O). While doing so, we quantify some potential errors and identify causes of variation in results from different laboratories, whether radioactive or stable isotopes are used.

After perturbation of metabolism with oral glucose, considerable variation exists among laboratories in both the cumulative and the peak rates of systemic glucose appearance (Table 1). Such differences arise even though variation in the results within laboratories
is relatively small, suggesting the occurrence of at least one systematic error. Using arterio-venous difference techniques, Ferrannini et al. (7), Pilo et al. (26), and Mari et al. (18) have shown that only ~5% of glucose is captured during its first pass through the splanchnic bed. Further capture of glucose for hepatic glycogen deposition must result from the 30–50 additional passes during the absorptive period. Consequently, the group mean end point for the appearance of glucose in the systemic circulation, given room for a ±5% error due to possible bias and sampling of the population, is expected to be between 90 and 100% by the time glucose absorption is complete. Values observed at present are 96 and 100% (Fig. 7) when the one-compartment model of Steele (33) and the two-compartment model of Mari et al. (16), respectively, are used. These agree with the observations by Radziuk and co-workers (28, 29), who found 93 and 92% with oral loads of 50 and 93 g glucose, respectively, when using radioactive tracers D-[3-3H]glucose intravenously and D-[1,14C]glucose orally. Other authors have also found cumulative systemic appearances in the range of 90–100%, notably on several different groups of volunteers in studies by Pehling et al. (23), who used mixed radioactive and stable isotopes, and Firth et al. (9), who used entirely radioactive isotopes (see Table 1).

Such expected systemic appearance of gut-derived glucose is not observed universally. Pilo et al. (26), Ferrannini et al. (7), Kelley et al. (11), Mittrakou and co-workers (20, 21), Kruszynska et al. (12), Mari et al. (18), and Delarue et al. (6) (see Table 1) all find cumulative systemic appearances of ~70%. Some authors have assumed that the discrepancy from the expected 100% absorption of glucose is a measure of first-pass splanchnic sequestration (20, 21), whereas others have accepted incomplete glucose absorption as the explanation of their results (7). Ferrannini et al. (7), Pilo et al. (26), and Mari et al. (18) demonstrate that their low recovery of oral D-[1,14C]glucose in systemic plasma, traced with intravenous D-[3-3H]glucose, cannot be due to first-pass splanchnic sequestration. Direct evidence of possibly incomplete absorption of glucose before the end of their experiments was not available. However, this seems an unlikely explanation for all of those studies with radioactive isotopes extending up to 5 or 6 h, in which cumulative Rnk glucose is still estimated to be as low as 70% of the oral dose (Table 1). Such unlikelihood is supported by the present stable isotope study, which indicates nearly complete absorption before 3.5 h (Fig. 7).

On the basis of observations we summarize from the literature (Table 1), no single explanation is apparent that will explain why some authors find theoretical systemic appearances of gut-derived glucose, whereas others find ~20% less. The glucose dose, the duration of the postprandial phase of the study, the balance of male and female volunteers, the existence of glucose intolerance and non-insulin-dependent diabetes, and the degree of obesity each offer no explanation. The analytic model, parameter assumptions, plasma sampling frequency at critical times, and the isotope combinations chosen are factors that might affect the precise estimate; however, individually, none is sufficiently influential to explain those published shortfalls in the cumulative systemic glucose appearance.

A partial explanation of the discrepancy (Table 1) is readily at hand for those studies in which the Steele or a similar model has been used with an assumption that the effective glucose distribution volume is in the range of 95–150 ml/kg body weight. These volumes were a priori assumptions taken from the early literature, where a different experimental situation existed, one that involved higher fluxes of intravenous glucose and insulin (to be discussed). The effective glucose distribution volume found at present is 230 ml/kg (Fig. 4). Should this value be reasonably accurate for other studies that use the current experimental situation (Table 1), we would expect underestimation of the cumulative glucose appearance by between 7 and 13%, which is approximately one-half of the 20% discrepancy noted.

Before discussing possible sources of the remaining discrepancy, we consider in the present and subsequent paragraphs the effective glucose distribution volumes adopted in the various studies (Vns in Table 1). In prior publications, volumes as low as 95–150 ml/kg body weight were adopted (rather than determined) for use with Steele’s (and equivalent) one-compartment model(s) applied to the oral loading dual tracer experiment. Such values originate from a total distribution space (VT), which varies from 200 ml/kg determined with mannitol and thiourea in dogs (10) to 260 ml/kg based on glucose in humans (33), and empirical observations that only a part (p) of this space seems to be accessible to glucose when metabolism is perturbed by either a rapid or a step change in intravenous glucose load or insulin injection (22). And so Vns in Table 1 equals pVT. The lower values of pVT in Table 1 (95–150 ml/kg) aimed to take account of the slow mixing of the infused glucose with the “extremities” of the total glucose distribution volume, as seemed to be apparent from intravenous infusion studies. However, a higher value would apply to the slower rates of entry of glucose from the gut than generally obtained in intravenous infusion experiments. With the change in the experimental situation, a different effective glucose pool would be expected, because the effective pool size is mass and time dependent (5, 10, 33). In the present work, we estimate neither VT nor p, however, when we assume that VT is independent of the experimental setup, the higher value for Vns (230 ml/kg) suggests that p must be large for the present type of study. High values of Vns due to high values of p are advantageous, because as p approaches unity, Vns loses time dependency.

It should be noted that the 230 ml/kg value is a study population mean value that we chose to use for all volunteers because variation in estimates among individuals was small (Fig. 4). Greater accuracy could result from the use of individually determined values of Vns, but we should recommend the use of more time intervals than used at present if this were to be done.
However, potential error due to application of a pooled value of $V_S$ is too small to be a major source of concern and cannot explain the large differences in results between laboratories.

Among the differences between laboratories in the estimates of cumulative glucose appearance (Table 1), model order appears not to be a significant factor. Radziuk and coworkers (28, 29) find high recoveries using a two-compartment model, whereas Ferrannini et al. (7) find low recoveries using the same model. Similarly, Mari et al. (18) find low recoveries (when using radioisotopes), whereas we find high recoveries (using stable isotopes) when the same two-compartment model is used. Nevertheless, model order may be viewed as important. Although Mari et al. showed that neither the one- nor the two-compartment model was demonstrably superior over the other for the assessment of glucose kinetics after oral glucose, it was also shown that considerable differences occur in rate estimates between models when $V_S$ in Steele's model is assumed to be 150 ml/kg. At present, we find such differences between models are largely eliminated when a value for $V_S$ of 230 ml/kg is used; indeed, the models yield very similar, or practically identical, information on $R_{aO}$ and $R_{ah}$ at each time interval. It is noteworthy that Pilo et al. (26) report a result for just one subject, which indicates comparable kinetics and recovery of oral glucose in plasma when one- and two-compartment models are used and $V_S$ is made large and possibly close to or equal to the “total glucose distribution space.” The far greater numbers of observations in the present study now make this particular observation robust. Clearly, marked differences in $R_{aO}$ and $R_{ah}$ between the one- and two-compartment models occur only when $V_S$ fails to balance the “volume” and “structure” errors suggested (5). The similarities in parameter estimates when $V_S = 230$ ml/kg support the adequacy for the present experimental situation of the larger glucose distribution volume found at present than that used previously (Table 1). Except for the confounding effects of an inappropriate $V_S$, model order does not explain a significant part of the large differences in literature values (Table 1) for cumulative systemic appearance of oral glucose.

A further outcome of using the higher glucose distribution volume with Steele's model is a plateau in the cumulative appearance of oral glucose beyond 3 h after glucose ingestion (Fig. 7). By trial, we found that too high a value of $V_S$ resulted in an overestimation of cumulative $R_{aO}$ initially and underestimation eventually, as summarized by the fractional sensitivity of rate estimates to $V_S$ in Fig. 8. The reverse is true for values of $V_s$ that are too small. A consequence is that an incorrect volume results in the absence of a plateau when glucose absorption is complete. It is noteworthy that when $V_S$ is too small, as in most cases (Table 1), absorption never appears to reach completion. Inspection of rate of appearance curves for oral glucose in several publications shows this to be a frequent occurrence (6, 9, 20, 21). Conversely, too high a value for $V_S$ results in peaking of the cumulative appearance curve, reaching too high values initially and false negative appearance rates eventually. Thus, in either case, the estimate of the cumulative appearance is then dependent on the duration over which glucose $R_a$ values are integrated, irrespective of when absorption is complete.

Both the present study and that of Delarue et al. (6) use D-[6,6-3H]glucose and D-[13C6]glucose species, but the results appear discrepant (Table 1). The present results cluster within studies from investigators using radioactive isotopes who find 90–100% systemic recovery of oral glucose, whereas those of Delarue et al. fall among those finding 70% recovery. Even after adjustment for the use of different effective glucose distribution volumes in the two studies, the results would still differ by 10% or more of the ingested glucose. The similar choice of isotopes indicates that incorrect assumptions about isotopomer metabolism are not the cause of the discrepancy. A possible source of variation is the frequency of plasma sampling for isotope analysis, particularly in the early period when absorption is rapid and changing. Table 1 indicates those published studies that sample plasma more frequently than every 30 min early after glucose ingestion (denoted F for frequent sampling) and those that do not (denoted I for infrequent sampling). With the study of Ferrannini et al. (7) and Mari et al. (18) being exceptional (see below), there is direct correspondence between early sampling frequency and result (Table 1), with the infrequent sampling resulting in an underestimation of cumulative systemic appearance of glucose. Furthermore, by close inspection of the one-compartment $R_{aO}$ time curve (Fig. 6), it can be seen that omission of a time point 15 min postingestion would result in a lower early $R_{aO}$ than that actually observed. The decrease in cumulative $R_{aO}$ would have been 9% for such an omitted sampling time. In addition, omission of the sample between 30 and 60 min would result in a further 5% error. The difference in estimated outcomes for the previous and the present dual stable isotope studies can therefore be explained by summation of a sampling frequency error and an effective volume error.

The two errors identified above, although sufficient to reconcile the majority of the study results, do not explain every difference. We have not positively identified the cause of the remaining differences, between Radziuk and coworkers (28, 29) and Ferrannini et al. (7) on the one hand and between the present results and those of Mari et al. (18) on the other, but we do not exclude sources of uncertainty, which may include incomplete absorption associated with the lower systemic appearances or the presence of a neutral, non-glucose radioactive contaminant in some preparations of commercial D-[3-3H]glucose (1). The purification of glucose during derivatization, gas chromatography, and selective mass spectrometry substantially reduces any risk of error due to contamination of stable isotopes of glucose.
The discussion so far has centered on possible explanations for too low values of oral glucose appearance in the systemic circulation. However, there are possible reasons why too high values could be observed because of the use of particular tracer isotopes. Thus Pehling et al. (23) and Firth et al. (9) used \( \text{d-}[2-3\text{H}] \) glucose intravenously in their mixed radioactive and stable dual isotope work. The loss of label from this isotopomer occurs from either glycogen synthesis or glycolysis (35) and so is expected to underestimate glucose utilization to a variable extent (30), pending the combined cycling rate of glucokinase and hexokinase. Appearance of oral glucose labeled with this isotopomer will be underestimated on this account, and this will offset an underestimate from use of a low \( V_S \); therefore, a result that is approximately correct is possible when these two errors cancel to yield the 92–93% recovery values (Table 1).

In conclusion, provided isotope recycling is minimal, as evident in the present study, stable isotopes are a suitable and safe alternative to radioactive isotopes for application in the dual isotope method with physiologically relevant oral glucose loads, at least in healthy humans. Sources of error in the methodology can occur whether radioactive or stable isotopes are used. Particular attention should be given both to the use of frequent plasma sampling, whatever model is used for data analysis, and to the larger effective glucose distribution volume when a single-compartment model is used. After such considerations, the differences in results for cumulative \( R_a \) among several laboratories are narrowed and favor the view that first-pass hepatic fractional sequestration of oral glucose is about the same as that for one-pass hepatic fractional sequestration of isotopic glucose. In studies involving less rapid changes in plasma glucose values, such as with certain starchy foods (14, 15), the use of the one-compartment model would seem even more justified than at present for glucose.

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