A critical evaluation of mass isotopomer distribution analysis of gluconeogenesis in vivo

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Previs, Stephen F., Gary W. Cline, and Gerald I. Shulman. A critical evaluation of mass isotopomer distribution analysis of gluconeogenesis in vivo. Am. J. Physiol. 277 (Endocrinol. Metab. 40): E154–E160, 1999.—There are conflicting reports concerning the reliability of mass isotopomer distribution analysis (MIDA) for estimating the contribution of gluconeogenesis to total glucose production (f) during [13C]glycerol infusion.1 We have evaluated substrate-induced effects on rate of appearance (R) of glycerol and glucose and f during [2-13C]glycerol infusion in vivo. Five groups of mice were fasted for 30 h and then infused with [2-13C]glycerol at variable rates and variable 13C enrichments (group I: 20 µmol·kg−1·min−1, 99% 13C; group II: 60 µmol·kg−1·min−1, 60% 13C; group III: 60 µmol·kg−1·min−1, 99% 13C; group IV: 120 µmol·kg−1·min−1, 40% 13C; or group V: 120 µmol·kg−1·min−1, 99% 13C). The total glycerol R was increased from ~104 to ~157 and to ~210 µmol·kg−1·min−1 as the infusion of [2-13C]glycerol increased from 20 to 60 and to 120 µmol·kg−1·min−1, respectively. As the amount of 99% enriched [2-13C]glycerol increased from 20 to 60 and to 120 µmol·kg−1·min−1 (groups I, III, and V, respectively), plasma glycerol enrichment increased from ~21 to ~42 and to ~57%, and the calculated f increased from ~27 to ~56 and to ~87%, respectively. Similar plasma glycerol enrichments were observed in groups I, II, and IV (i.e., ~21–24%), yet f increased from ~27 to ~57 and to ~86% in groups II and IV, respectively. Estimates of absolute gluconeogenesis increased from ~14 to ~33 and ~86 µmol·kg−1·min−1 as the infusion of [2-13C]glycerol increased from 20 to 60 and 120 µmol·kg−1·min−1, respectively. Plausible estimates of f were obtained only under conditions that increased total glycerol R by 2-fold (P < 0.001) and increased glucose R by 1.5-fold (P < 0.01) above basal. We conclude that in 30-h fasted mice, 1) estimates of f by MIDA with low infusion rates of [2-13C]glycerol yield erroneous results and 2) reasonable estimates of f are obtained at glycerol infusion rates that perturb glycerol and glucose metabolism.

stable isotopes; mass spectrometry; triose phosphate turnover; diabetes; mice

STRONG ET AL. (29) originally reported that it is possible to measure the biosynthesis of polymeric molecules if a labeled precursor is administered and the mass isotopomer distribution of the polymer is determined. More recently, mass isotopomer distribution analysis (MIDA) was proposed as a method for estimating the fractional synthetic rate of various biopolymers including cholesterol, fatty acids, glucose, and DNA (11). (For a comprehensive review of the application of isotopomer analysis methods for studying physiological problems see Ref. 3.)

Glucose can be considered as a dimer formed from the condensation of two triose phosphate molecules. Thus MIDA of glucose made from a 13C-labeled gluconeogenic precursor(s) has been proposed as a method for estimating the contribution of gluconeogenesis to total glucose production (f; Refs. 20, 21). MIDA permits the determination of the triose phosphate enrichment. In contrast to other tracer methods, MIDA calculations of f are not subject to artifacts of isotope exchange or dilution, which have limited investigations of gluconeogenesis (5, 14, 15). The main underlying assumption of MIDA, however, is that the triose phosphate pool(s) in all gluconeogenic cells must be at similar 13C enrichments (16, 24, 25). If this assumption is not valid, f is underestimated (16, 24, 25).

There are conflicting opinions regarding the general applicability of MIDA for estimating f during the infusion of [13C]glycerol in vivo. Investigators have infused [2-13C]glycerol and concluded that it is possible to correctly estimate f (12, 20, 21, 23). However, other investigators have infused [U-13C3]glycerol and concluded that f was underestimated and that MIDA is not a reliable method for estimating f (16, 24). A recent report (25) has shown that [2-13C]glycerol and [U-13C3]glycerol provide the same estimation of f if used under identical conditions in isolated hepatocytes. In addition, this in vitro study demonstrated that the relative contribution of 13C glycerol vs. other gluconeogenic precursors plays a role in determining f, such that f increased as the contribution of 13C glycerol increased (25). Lastly, the hepatocyte experiments demonstrated that glucose production increased as the supply of glycerol increased. Thus it appears that the amount of [13C]glycerol infused, and not the type of 13C glycerol, may be an important factor for applying MIDA to studies of gluconeogenesis.

Because of the increasing application of the MIDA technique (7, 10, 13, 28, 30) and the uncertainty of its reliability in vivo (16, 22, 24), we have asked two questions. First, are there substrate-induced effects on glycerol and glucose metabolism during [2-13C]glycerol infusion in vivo? Second, do substrate-induced effects have an impact on MIDA and estimations of f?

MATERIALS AND METHODS

Materials. Unless noted, chemicals were purchased from Sigma-Aldrich. [2-13C]Glycerol (99 atom percent excess) was purchased from Cambridge Isotopes (Andover, MA). [3-3H]glu-
cose was purchased from New England Nuclear (Boston, MA). Gas chromatography-mass spectrometry (GC-MS) supplies were purchased from Hewlett-Packard (Wilmington, DE). N-methyl-N-(tert-butyl)dimethylsilyl trifluoroacetamide + 1% tert-butylidimethylchlorosilane (TBDMs) and bis(trimethylsilyl)trifluoroacetamide + 10% trimethylchlorosilane (TMS) were purchased from Pierce (Rockford, IL).

In vivo studies. Male BALB/C mice (Charles River, 20–25 g) were fitted with jugular vein catheters and allowed a 4-day recovery. Mice were fasted 30 h and infused with [2-13C]glycerol at variable rates and variable 13C enrichments (group I: 20 µmol·kg⁻¹·min⁻¹, 99% 13C; group II: 60 µmol·kg⁻¹·min⁻¹, 60% 13C; group III: 60 µmol·kg⁻¹·min⁻¹, 99% 13C; group IV: 120 µmol·kg⁻¹·min⁻¹, 40% 13C; or group V: 120 µmol·kg⁻¹·min⁻¹, 99% 13C). [3-1H]glucose was infused at 1.0 µmol·kg⁻¹·min⁻¹ to estimate total glucose production.

During preliminary experiments (Table 1; Fig. 1), blood (~65 µl) was sampled at 0, 60, 100, 140, and 180 min. These studies revealed that the plasma glycerol enrichment and calculated values of f reached a steady state before 140 min. During all additional studies, blood (~65 µl) was sampled via tail-tip bleeds at 0, 140, and 180 min for measurement of the concentration and labeling of glycerol, glucose, and lactate.

Analyses. Plasma samples obtained at 140 and 180 min were processed for 3H counting as follows. Briefly, a 5-µl aliquot of plasma was deproteinized by adding 10 µl of 10% trichloroacetic acid. A 10-µ1 portion of the supernatant was dried to remove 3H2O. The residue was dissolved in 100 µl of water. After addition of 2 ml of scintillation fluid (Ultima Gold, Packard Instrument, Meriden, CT), samples were counted for 1 min.

GC-MS analyses were conducted with a Hewlett Packard 5973 MSD equipped with a Hewlett Packard 6890 GC. All samples were analyzed with split injection (10:1) on a Hewlett Packard 5MS capillary column (30 m 0.25 mm 0.25 µm) maintained at a constant helium flow (1.2 ml/min). Plasma samples obtained at 0, 140, and 180 min were processed for GC-MS analysis of the mass isotopomer distribution of glucose as follows. A 10-µl plasma sample was added to 100 µl of 100% methanol. The supernatant was evaporated to dryness. The mass isotopomer distribution of glucose was determined after conversion to its aldonitrile pentaacetate derivative with chemical ionization (methane gas). This sample was also analyzed with electron impact ionization to determine 13CO2 reincorporation in one-half of the glucose (2, 19). 13CO2 reincorporation could lead to the formation of a double-labeled triose phosphate, and if uncorrected for, gluconeogenesis would be underestimated (19). We did not find any M + 2 Glc in the fragment corresponding to C4 to C6 Glc.

We determined the concentration of glucose and the enrichment and concentration of glycerol and lactate as follows. Briefly, a known amount of [U-13C3]glucose, [3-1H]glycerol, and [U-13C3]lactate was added to 25 µl of plasma. The sample was added to 200 µl of 100% methanol. The supernatant was split into three parts (150, 30, and 30 µl), and each was evaporated to dryness. Plasma glycerol concentration and 13C enrichment were determined on the 150-µl fraction, as its TBDMS derivative with electron ionization. The glycerol concentration was calculated from the mass-to-charge ratio (m/z) 206/206 signal, and the glycerol enrichment was calculated from the m/z 308/308 signal. A 30-µl fraction was used to determine the concentration of glucose, as its aldonitrile pentaacetate derivative with chemical ionization. The glucose concentration was calculated from the m/z 328/328 and 330/330 signal. The second 30-µl fraction was used to determine the concentration and 13C enrichment of plasma lactate, as its TBDMS derivative with electron ionization conditions. The lactate concentration was calculated from the m/z 262/262 signal, and the lactate enrichment was calculated from the m/z 262/262 signal.

All samples were analyzed in duplicate. Background mass isotopomer distributions were corrected (9, 27). Data are presented as the means ± SE. Statistics were calculated with one-way ANOVA, and Tukey’s post hoc testing was used to determine significance.

Calculations. The total rate of appearance (Ra) of glycerol, in µmol·kg⁻¹·min⁻¹, equals

\[
\frac{[2-13C]glycerol\ infusion\ rate\ (\mu mol\ ·\ kg^{-1}·\ min^{-1})}{plasma\ glycerol\ 13C\ enrichment}\ 
\]

The endogenous Ra of glycerol, in µmol·kg⁻¹·min⁻¹, equals

\[
\text{total glycerol } Ra - \text{glycerol infusion rate}
\]

The Ra of glucose, in µmol·kg⁻¹·min⁻¹, equals

\[
\frac{[3-1H]glucose\ infusion\ rate\ (dpm·kg^{-1}·min^{-1})}{plasma\ glucose\ specific\ activity\ (dpm/\mu mol)}
\]

The fractional triose phosphate enrichment equals

\[
\frac{2 \times \frac{M + 2\text{Glc}}{M + \text{Glc}}}{1 + 2 \times \frac{M + 2\text{Glc}}{M + \text{Glc}}}
\]
where \( M + 1_{\text{glucose}} \) and \( M + 2_{\text{glucose}} \) are the molar percent excess values of glucose with one and two \(^{13}\text{C} \) atoms, respectively. The contribution of gluconeogenesis to \( f \) equals

\[
\frac{M + 1_{\text{glucose}}}{2p(M - p)} = \frac{M + 2_{\text{glucose}}}{p^2}
\]

where \( p \) is the fractional triose phosphate enrichment.

The absolute rate of gluconeogenesis, in \( \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \), equals

\[
\text{glucose } R_a \times f
\]

RESULTS

Data from preliminary studies revealed that steady-state concentrations and enrichments of plasma glycerol were observed under the conditions tested. For example, Fig. 1 shows the profile of plasma glycerol concentration and \(^{13}\text{C} \) enrichment in mice infused with \([2-^{13}\text{C}]\)glycerol (20 \( \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \), 99% \(^{13}\text{C} \)). Similar steady-state data were obtained from mice infused at the other rates of glycerol (not shown).

Table 1 shows the mass isotopomer distribution of plasma glucose and the contribution of gluconeogenesis to total glucose production from 100 to 180 min in mice infused with \([2-^{13}\text{C}]\)glycerol (20 \( \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \), 99% \(^{13}\text{C} \)). Calculated values of \( f \) were in steady state by 100 min. Similar steady-state data were obtained from mice infused at the other rates of glycerol (not shown).

Table 2 shows the effect of increasing glycerol infusion to 30-h fasted mice. Data were averaged from samples obtained at 140 and 180 min. Despite a threefold increase in plasma glycerol concentration and a twofold increase in total glycerol \( R_a \), when the glycerol infusion rate was increased from 20 to 120 \( \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \), the endogenous glycerol \( R_a \) was similar in each group. Thus the increase in total glycerol \( R_a \) was induced by the exogenous glycerol infusion. As the glycerol infusion increased from 20 to 120 \( \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \), the plasma lactate enrichment increased from 0.583 \pm 17.5 \( r^2 = 0.98 \) and absolute gluconeogenesis \( (y = 0.732x - 4.53; r^2 = 0.97) \) as the infusion rate of glycerol increased (Fig. 2). These data demonstrate that estimates of gluconeogenesis are dependent on the load of glycerol and independent of the calculated triose phosphate enrichment.

Table 4 shows the effects of variable \([2-^{13}\text{C}]\)glycerol infusion on plasma lactate \(^{13}\text{C} \) enrichment and concentration. As the infusion rate of 99% enriched \([2-^{13}\text{C}]\)glycerol increased from 20 to 60 and to 120 \( \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \), plasma lactate enrichment increased from \( \sim9 \) to \( \sim19 \) and to \( \sim30 \), respectively. Plasma lactate enrichment did not change in the groups I, II, and IV, which had similar plasma glycerol enrichments. Lactate concentration remained relatively constant in all groups.

DISCUSSION

In theory, after the infusion of a \(^{13}\text{C} \)-labeled gluconeogenic precursor (e.g., \([^{13}\text{C}]\)glycerol), the MIDA of plasma glucose can be used to estimate contribution of gluconeogenesis to \( f \) (11). An example may clarify how MIDA works for calculating \( f \). Consider the synthesis of glucose during the infusion of \([2-^{13}\text{C}]\)glycerol in vivo. The triose phosphate pool contains species at 0, 1, 2, and 3, respectively. From the ratio of \( M + 2_{\text{glucose}} \) to \( M + 1_{\text{glucose}} \) one can infer the triose phosphate enrichment (i.e., Eq. 4) and then calculate \( f \) (i.e., Eq. 5). If \([U-^{13}\text{C}_2]\)glycerol is infused, the triose phosphate pool contains species at \( M + 0 \) to \( M + 3 \) and newly synthesized glucose contains species at \( M + 0 \) to \( M + 6 \) (16, 24, 25).

Table 2. Substrate-induced effects of increasing glycerol infusion on glycerol and glucose metabolism in 30-h fasted mice infused with variable amounts of \([2-^{13}\text{C}]\)glycerol

<table>
<thead>
<tr>
<th>Group</th>
<th>Infusion rate, ( \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} )</th>
<th>Enrichment, MPE</th>
<th>Plasma Glycerol</th>
<th>Plasma Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Enrichment, MPE</td>
<td>Concentration, ( \mu\text{M} )</td>
</tr>
<tr>
<td>I</td>
<td>20</td>
<td>99</td>
<td>21 ± 3</td>
<td>388 ± 15</td>
</tr>
<tr>
<td>II</td>
<td>60</td>
<td>60</td>
<td>24 ± 2</td>
<td>716 ± 23*</td>
</tr>
<tr>
<td>III</td>
<td>60</td>
<td>99</td>
<td>42 ± 5*</td>
<td>724 ± 25*</td>
</tr>
<tr>
<td>IV</td>
<td>120</td>
<td>40</td>
<td>24 ± 3</td>
<td>1,359 ± 59*</td>
</tr>
<tr>
<td>V</td>
<td>120</td>
<td>99</td>
<td>57 ± 5*</td>
<td>1,392 ± 48*</td>
</tr>
</tbody>
</table>

Values are means ± SE; \( n = 6-12 \). \( R_a \), rate of appearance. *\( P < 0.001 \) vs. group I; †\( P < 0.001 \) for group II vs. IV or III vs. V.
described above, although the equations are more complex and generally require a computer algorithm to solve for the triose phosphate mass isotope distribution (16, 24, 25).

Currently, there are contrasting opinions regarding the validity of MIDA for estimating f during [13C]glycerol infusion in vivo. Investigators have infused [2-13C]glycerol and concluded that it is possible to correctly estimate f (20, 21, 23). However, other investigators have infused [U-13C3]glycerol and concluded that f was underestimated (16, 24). It was proposed that the apparent underestimation of f could be related to a transplanchic (or transhepatic) decrease in the concentration and enrichment of [13C]glycerol (16). Such gradients were directly measured with arteriovenous isotope balance measurements (17, 24, 26).

Data from the study of Ekberg et al. (8) indirectly support the stable isotope data demonstrating a transplanchic gradient of glycerol concentration and enrichment in vivo (17, 24, 26). Briefly, 36-h-fasted subjects ingested 0.5 g of acetaminophen and were infused with [2-14C]glycerol and [1-14C]lactate. The 14C distribution in blood glucose and urinary acetaminophen glucuronide was measured. [2-14C]glycerol labels C-2 and C-5 of blood glucose and urinary glucuronide. [1-14C]lactate labels C-3 and C-4 of blood glucose and urinary glucuronide. The labeling ratio (C-2 + C-5) to (C-3 + C-4) was higher in the blood glucose than in the urinary glucuronide. Presumably, the acetaminophen sampled a hepatic pool of glucose 6-phosphate in which there was more label from lactate than from glycerol. It was concluded that the concentration of glycerol decreased much faster than the concentration of lactate as blood passed from perportal to perivenous hepatocytes.

Table 3. Mass isotopomer distribution of plasma glucose after variable infusion rates of [2-13C]glycerol

<table>
<thead>
<tr>
<th>Group</th>
<th>Infusion rate, μmol·kg⁻¹·min⁻¹</th>
<th>Enrichment, MPE</th>
<th>Glucose Mass Isotopomer Distribution, MPE</th>
<th>Triose Phosphate Enrichment, MPE</th>
<th>Gluconeogenesis, f, %</th>
<th>μmol·kg⁻¹·min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>20</td>
<td>99</td>
<td>90.0 ± 1.6</td>
<td>15 ± 2</td>
<td>27 ± 3</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>II</td>
<td>60</td>
<td>60</td>
<td>83.8 ± 1.8</td>
<td>14 ± 2</td>
<td>57 ± 3</td>
<td>33 ± 2</td>
</tr>
<tr>
<td>III</td>
<td>60</td>
<td>99</td>
<td>75.3 ± 2.1</td>
<td>23 ± 2*</td>
<td>56 ± 3</td>
<td>34 ± 5</td>
</tr>
<tr>
<td>IV</td>
<td>120</td>
<td>40</td>
<td>75.3 ± 1.4</td>
<td>15 ± 1</td>
<td>86 ± 2*</td>
<td>84 ± 10*</td>
</tr>
<tr>
<td>V</td>
<td>120</td>
<td>99</td>
<td>39.7 ± 2.5</td>
<td>45 ± 3*†</td>
<td>87 ± 2*</td>
<td>88 ± 12†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6–12. Shown are calculated triose phosphate enrichments and f for each group. *P < 0.001 vs. group I; †P < 0.001 for group II vs. IV or III vs. V.

More recently, in vitro studies demonstrated that MIDA estimates of f are dependent on the relative flux of [13C]glycerol vs. other gluconeogenic substrates and independent of the type of [13C]glycerol infused (25). This raised the question as to whether estimates of f are affected by increasing the infusion rate of [2-13C]glycerol in vivo. We found that as the infusion rate of [2-13C]glycerol increased from 20 to 60 and to 120 μmol·kg⁻¹·min⁻¹ that estimates of f increased from ~27 to 56 and to 86%, respectively (Table 3). These findings suggest that not all gluconeogenic cells are equally labeled when low doses of [2-13C]glycerol are infused. Presumably, as the infusion rate of [2-13C]glycerol increased, a more homogeneous gluconeogenic precursor enrichment was achieved. In addition, as the infusion rate of [2-13C]glycerol increased from 20 to 120 μmol·kg⁻¹·min⁻¹, estimates of total glucose production increased from ~63 to ~105 μmol·kg⁻¹·min⁻¹, respectively. In this study, estimates of absolute gluconeogenesis increased from ~14 to ~33 and to ~86 μmol·kg⁻¹·min⁻¹, as the infusion rate of [2-13C]glycerol increased from 20 to 60 and to 120 μmol·kg⁻¹·min⁻¹, respectively (Table 3).

Table 4. Effect of variable [2-13C]glycerol infusion on plasma lactate concentration and enrichment

<table>
<thead>
<tr>
<th>Group</th>
<th>Infusion rate, μmol·kg⁻¹·min⁻¹</th>
<th>Enrichment, MPE</th>
<th>Concentration, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>20</td>
<td>99</td>
<td>9.4 ± 0.90</td>
</tr>
<tr>
<td>II</td>
<td>60</td>
<td>60</td>
<td>12.0 ± 2.12</td>
</tr>
<tr>
<td>III</td>
<td>60</td>
<td>99</td>
<td>18.8 ± 0.56*</td>
</tr>
<tr>
<td>IV</td>
<td>120</td>
<td>40</td>
<td>12.3 ± 0.98</td>
</tr>
<tr>
<td>V</td>
<td>120</td>
<td>99</td>
<td>30.0 ± 3.78*†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6. *P < 0.001 vs. group I; †P < 0.001 for group II vs. IV or III vs. V.

Fig. 2. Estimates of gluconeogenesis during variable glycerol infusion rates in 30-h fasted mice. Contribution of gluconeogenesis in mice infused with 60 μmol [2-13C]glycerol·kg⁻¹·min⁻¹ (either 60 or 99% [13C]glycerol·kg⁻¹·min⁻¹) or with 120 μmol [2-13C]glycerol·kg⁻¹·min⁻¹ (either 40 or 99% [13C]glycerol·kg⁻¹·min⁻¹) increased from that calculated for mice infused with 20 μmol [2-13C]glycerol·kg⁻¹·min⁻¹ (99% [13C]). This is true whether one calculates f (y = 0.583x + 17.5; r² = 0.98) or estimates absolute gluconeogenesis (y = 0.732x – 4.53; r² = 0.97).
MIDA of gluconeogenesis is limited to the extent that the \(^{13}\text{C}\) gluconeogenic precursor does not enter all gluconeogenic cells equally. Metabolic zonation of gluconeogenesis can lead to heterogeneity in labeling the triose phosphate pool. Several mechanisms have been presented by which metabolic zonation can occur (8, 16, 17, 24–26). These include 1) a decrease in substrate concentration across a gluconeogenic organ, 2) dilution of tracer across a gluconeogenic organ, 3) inflow of substrate close to or below the Michaelis-Menten constant (Km) of the first rate-limiting enzyme that acts on the substrate, 4) multiple gluconeogenic organs, and/or 5) relative intracellular flux into the triose phosphate pool is not constant in all gluconeogenic cells. In particular, metabolic zonation of the triose phosphate labeling can be induced by transhepatic changes in the concentrations of various gluconeogenic precursors (e.g., glycerol and lactate). Most likely, in previous studies, periportal hepatocytes were exposed to more \([U-^{13}\text{C}_3]\)glycerol than perivenous hepatocytes (16, 24). As a result, gluconeogenesis occurring in the perivenous zone of the liver was erroneously ascribed to glycogenolysis. Similar substrate gradients may develop across the kidney (17, 26) and could lead to heterogeneity of renal glucose production. Renal glucose production may be important during some conditions (1, 4, 6, 22). Presumably, in our current study the increase in total glycerol concentration must have resulted in blunting of transhepatic (and transrenal) gradients in the concentration and \(^{13}\text{C}\) enrichment of glycerol. Consequently, a more homogeneous triose phosphate enrichment was maintained.

The groups of Hellerstein and co-workers (Dekker et al. (7) and Siler et al. (28)) and Peroni et al. (23) have suggested that perhaps methodological error(s) was responsible for the underestimation of f when \([U-^{13}\text{C}_3]\)glycerol was infused in vivo. However, in previous studies the mass isotopomer distributions of blood glucose and urinary acetaminophen glucuronide (analyzed as glucose) from humans infused with \([U-^{13}\text{C}_3]\)glycerol were determined by independent laboratories with different derivatization techniques and analytical conditions (16). Despite the use of different methodologies, the calculated f values were similar and unexpectedly low (16). This indirectly argues against the occurrence of systematic error related to the use of \([U-^{13}\text{C}_3]\)glycerol.

Recently, Dekker et al. (7) stated that “the use of \([U-^{13}\text{C}_3]\)glycerol and \([U-^{13}\text{C}_3]\)lactate leads to predictable problems due to enormous dynamic range for abundances of \(M_{-6}\) compared to \(M_{0}\); \(M_{-1}\)… the ratio of \(M_{-6}/M_{0}\) with \([U-^{13}\text{C}_3]\)glycerol or \([U-^{13}\text{C}_3]\)lactate is greater than 1,000:1…”. Although calculations of f are sensitive to analytical error and the use of \(U-^{13}\text{C}_3\)-labeled tracers requires a complex data fitting algorithm, the statement made by Dekker et al. (7) is unfounded. When one more closely compares the \(M_{-6}/M_{0}\) ratio with estimates of f, it is clear that there is no correlation between the \(M_{-6}/M_{0}\) ratio and estimates of f (24). For example, the largest \(M_{-6}/M_{0}\) ratios can be found in the in vivo studies (i.e., rats or monkeys; Ref. 24). Glucose from rats infused with \([U-^{13}\text{C}_3]\)glycerol had a \(M_{-6}/M_{0}\) ratio of −686:1 and f was −75%. Glucose from rats infused with \([U-^{13}\text{C}_3]\)lactate had a \(M_{-6}/M_{0}\) ratio of −1,696:1, and f was −97%. Thus, under identical experimental conditions in rats, the greatest \(M_{-6}/M_{0}\) ratio was observed during \([U-^{13}\text{C}_3]\)lactate infusion; yet, f was in the expected range considering the nutritional status (i.e., 48 h fasted). In monkeys, the \(M_{-6}/M_{0}\) ratio was similar during \([U-^{13}\text{C}_3]\)glycerol or \([U-^{13}\text{C}_3]\)lactate infusion, yet the f calculated during \([U-^{13}\text{C}_3]\)lactate infusion was approximately twofold higher than f calculated during \([U-^{13}\text{C}_3]\)glycerol infusion (80 vs. 48%). Furthermore, the value calculated for f was independent of the type of \([^{13}\text{C}]\)lactate infused because similar f values were calculated in monkeys infused with \([U-^{13}\text{C}_3]\)lactate (f −80%) or \([3-^{13}\text{C}]\)lactate (f −81%; see Ref. 25). The f calculated during the infusion of \([^{13}\text{C}]\)lactate to monkeys is reasonable considering their nutritional status (18 h fasted). Also, Lee et al. (18) obtained reasonable estimates of f during the infusion of \([^{13}\text{C}]\)lactate to humans.

From the accumulated data (8, 16, 17, 24–26), one can conclude that a possible cause of the discrepancy in the estimates of f observed at various glycerol infusion rates may lie not in the isotopomer ratios and analytical techniques but in the substrate concentrations. In fact, with anesthetized rats, Peroni et al. (21) concluded that accurate in vivo measurements of f could be made at the expense of some perturbation of the metabolic pathway studied. In our study, we attempted to estimate gluconeogenesis at variable \([2-^{13}\text{C}]\)glycerol infusion rates and independent of triose phosphate labeling. We found \([2-^{13}\text{C}]\)glycerol infusion rates that maintained plasma glycerol enrichments at approximately the same level (i.e., 21–24%; groups I, II, and IV) and allowed us to give increasing quantities of glycerol. When either 60 or 99% enriched \([2-^{13}\text{C}]\)glycerol was infused at 60 \(\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}\), we observed that the plasma glycerol concentration increased approximately twofold over basal (Table 2). However, despite the different plasma glycerol enrichments (24 vs. 42%) and calculated triose phosphate enrichments (14 vs. 23%), similar values of f were calculated (57 vs. 56%). Likewise, similar metabolic effects were observed when either 40 or 99% enriched \([2-^{13}\text{C}]\)glycerol was infused at 120 \(\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}\) (Table 2). Different plasma glycerol enrichments were imposed (24 vs. 57%), and different triose phosphate enrichments were calculated (15 vs. 45%), yet similar values of f were calculated (86 vs. 87%). Our findings indicate that by increasing the infusion rate of \([2-^{13}\text{C}]\)glycerol from 20 to 120 \(\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}\) that estimates of f increased from −27 to −87%, respectively. The increase in f was independent of the calculated triose phosphate enrichment (i.e., groups I, II, and IV). These data show that calculations of f were apparently limited by the supply of glycerol.

Lastly, our findings point to another potential limitation of MIDA regarding the calculation of the triose phosphate \(R_a\). Neese et al. (20) proposed that it is possible to estimate the triose phosphate \(R_a\) during the
infusion of [2-13C]glycerol. However, to calculate the triose phosphate $R_a$ one must assume that [2-13C]glycerol is only taken up by the liver and that no other 13C-labeled compounds contribute to the influx to triose phosphates (20). Others have demonstrated that extrahepatic glycerol utilization is significant (17, 26) and that plasma lactate becomes labeled during the infusion of [U-13C]glycerol (24). Under the conditions tested here, we found that plasma lactate becomes significantly enriched during the infusion of [2-13C]glycerol (Table 4). In particular, the lactate enrichment increased with increasing amounts of 99% enriched [2-13C]glycerol. Thus our findings demonstrate that it is not possible to directly calculate the triose phosphate $R_a$, because one must account for [13C]lactate flux to the triose phosphate pool.

On the basis of results of this study and earlier studies, we conclude that MIDA with [2-13C]glycerol is not a reliable method for estimating $f$. Estimates of $f$ by MIDA with low infusion rates of [2-13C]glycerol yield erroneous results. Errors in MIDA are most likely due to metabolic zonation of gluconeogenesis, which leads to heterogeneity in labeling the triose phosphate pool. Presumably, transhepatic (and transrenal) glycerol gradients are decreased during high glycerol flux, and therefore a more homogeneous triose phosphate enrichment is maintained. Could there be conditions of increased glycerol flux during which the infusion of [13C]glycerol and MIDA would provide reliable estimates of gluconeogenesis? One might expect an increased glycerol flux during total parenteral nutrition with lipid emulsions. However, detailed studies of MIDA estimates of gluconeogenesis have not yet been performed under such conditions.

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