Loss of $^{13}$C Glycerol Carbon via the Pentose Cycle

IMPLICATIONS FOR GLUCONEOGENESIS MEASUREMENT BY MASS ISOTOPER DISTRIBUTION ANALYSIS*

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Whereas many reports substantiated the suitability of using [2-$^{13}$C]glycerol and Mass Isotoper Distribution Analysis for gluconeogenesis, the use of [$^{13}$C]glycerol had been shown to give lower estimates of gluconeogenesis (GNG). The reason for the underestimation has been attributed to asymmetric isotope incorporation during gluconeogenesis as well as zonation of gluconeogenic enzymes and a [$^{13}$C]glycerol gradient across the liver. Since the cycling of glycerol carbons through the pentose cycle pathways can introduce asymmetry in glucose labeling pattern and tracer dilution, we present here a study of the role of the pentose cycle in gluconeogenesis in Fao cells. The metabolic regulation of glucose release and gluconeogenesis by insulin was also studied. Serum-starved cells were incubated for 24 h in Dulbecco’s modified Eagle’s media containing 1.5 mM [U-$^{13}$C]glycerol. Mass isotopomers of whole glucose from medium or glycogen and those of the C-1—C-4 fragment were highly asymmetrical, typical of that resulting from the cycling of glucose carbon through the pentose cycle. Substantial exchange of tracer between hexose and pentose intermediates was observed. Our results offer an alternative mechanism for the asymmetrical labeling of glucose carbon from triose phosphate. The scrambling of $^{13}$C in hexose phosphate via the pentose phosphate cycle prior to glucose release into the medium is indistinguishable from dilution of labeled glucose by glycojen using MIDA and probably accounts for the underestimation of GNG using $^{13}$C tracer methods.

In the liver, the phosphorylation of glycerol and its subsequent conversion to glyceraldehyde phosphate (GAP) and dihydroxyacetone phosphate (DHAP) provides the basis of an important isotopomer method for the determination of gluconeogenesis using $^{13}$C-labeled glycerol (1). Whereas many reports substantiated the suitability of using [2-$^{13}$C]glycerol and MIDA for gluconeogenesis (2, 3), the use of [U-$^{13}$C]glycerol had been shown to give lower estimates of gluconeogenesis (4). The reason for the underestimation has been attributed to the cycling of triose phosphate and glycerol and the lack of complete equilibrium between GAP and DHAP leading to asymmetric isotope incorporation as well as zonation of gluconeogenic enzymes and a [$^{13}$C]glycerol gradient across the liver during gluconeogenesis. How these different factors may affect the precision and accuracy of the MIDA approach in the estimation of gluconeogenesis has not been demonstrated (1, 4, 5).

When rat liver was perfused after a 2-day fast with [U-$^{13}$C]glycerol, M1 and M2 glucose mass isotopomers were found in addition to the M3 and M6 of glucose predicted on the basis of the combination of two m3 triose-P molecules. Since M1 and M2 isotopomers were also seen in triose-P and in phosphoenolpyruvate (PEP) (4), the source of M1 and M2 isotopomers of glucose was assumed to come from the loss of carbon via the tricarboxylic acid cycle. Since glyceraldehyde phosphate is also an intermediate of the pentose cycle, the cycling of glycerol carbons through the pentose cycle pathways produces asymmetry in glucose carbon labeling pattern and can also lead to the formation of M1 and M2 glucose. We present here a study of the role of the pentose cycle in gluconeogenesis in Fao cells, in which the cycling of [U-$^{13}$C]glycerol carbon between glycerol and PEP was minimized with the dilution of PEP from unlabeled gluconeogenic precursors, pyruvate and glutamine. Fao hepatoma cells are derived from the Reuber H35 rat hepatoma cell line (6, 7) and show stable expression of a number of liver-specific functions including the expression of the gluconeogenic enzymes PEP carboxykinase and fructose 1,6-bisphosphatase. Since Fao cells can grow in glucose-free media and have a uniform enzymatic profile, unlike the mixture of perportal and perivenous cells seen in primary hepatocyte culture, Fao cells were used to study gluconeogenesis. In addition, Fao cells have been found to have specific and high affinity binding of insulin at physiologic concentrations. The metabolic regulation of glucose release and gluconeogenesis by insulin was also studied.

EXPERIMENTAL PROCEDURES

Materials—The Fao hepatoma cell line was obtained from the American Tissue Culture Collection (ATCC). [1,2,3-$^{13}$C$_3$]Glycerol or [U-$^{13}$C]glycerol (>99% enriched) was purchased from Isotec (Miamisburg, OH).

Tissue Culture Conditions—The Fao cells were serum-starved for 18 h in low glucose (1 g/liter) DMEM, containing an additional 1 mM glutamine, 1 mM sodium pyruvate, and 1% penicillin/streptomycin before the isotope study. The medium was then changed to DMEM with no glucose, which contains sodium pyruvate, 1 mM glutamine (2 mM final), and penicillin/streptomycin as in the low glucose condition. Dexamethasone was then added to a final concentration of 10 $^{-6}$ M, and the final concentration of the ETOH carrier for dexamethasone was 0.02%. Two substrate conditions (2 mM xylitol or 12.5 mM glucose) and two hormone conditions (in the presence or absence of insulin) were used giving rise to four incubation conditions. Undiluted [U-$^{13}$C]glycerol was added to

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§ The abbreviations used are: GAP, glyceraldehyde phosphate; DHAP, dihydroxyacetone phosphate; PEP, phosphoenolpyruvate; DMEM, Dulbecco’s modified Eagle’s medium; EI, electron impact ionization; CI, chemical ionization; G6PDH, glucose-6-phosphate dehydrogenase; PPP, pentose phosphate pathway; MIDA, Mass Isotoper Distribution Analysis.

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36787
all culture plates to a final [U-13C]glycerol of 1.5 mM to start the experiment. For incubation with insulin, the cells were stimulated with dexamethasone for 2 h before the addition of insulin. The final concentration of insulin was 10⁻⁶ M. The plates were incubated for a total of 24 h after the time the media were collected.

**Sample Processing.** At the end of experiment, cells were harvested in ice-cold phosphate-buffered saline, spun down, resuspended in a total volume of 50 μl with phosphate-buffered saline, and stored at −80 °C until processed. This pellet was sonicated prior to measurement of glycogen or ribose. Glycogen glucose was isolated from cell pellets after treatment with amyloglucosidase (8). RNA was extracted from cell pellets after treatment with ice-cold Trizol 1:2, v/v (9, 10). Incubation medium from each culture plate was analyzed for lactate, glucose, and glutamate using previously established methods. Lactate was first extracted from the acidified medium using ethyl acetate. Glucose, glutamate, and glutamate were separated using ion-exchange chromatography. (8) Glucose and xylitol were co-eluted in the neutral fraction from the medium.

**Gas Chromatography-Mass Spectrometry Analysis—Medium or glycogen glucose was derivatized as its penta-acetate or aldonitrile penta-acetate derivative for gas chromatography-mass spectrometry analysis (11). Under either derivatization condition, xylitol was converted to its acetate derivative by the acetic anhydride reaction. GC conditions were as follows: capillary column (HP8); carrier gas, helium at flow rate of 1 ml/min; temperature 220 °C for 2 min and then a temperature gradient of 10 °C per min until final temperature of 250 °C; xylitol penta-acetate and glucose penta-acetate were separated. Chemical ionization (CI) was used to give the molecular ion (C-1—C-6) of the glucose molecule at m/z 331 for its penta-acetate and m/z 328 for its aldonitrile penta-acetate. Electron impact ionization (EI) of the aldonitrile derivative was used to characterize glucose positional isotopomers at m/z 187 for C-3—C-6 and m/z 242 for C-1—C-4 fragments. Ribose was converted to its aldonitrile derivative and lactate to its heptafluorobutyrate aldonic derivative and lactate to its heptafluorobutyrate n-propylamine derivatized as described previously (12).

Results of the mass isotopomers in glucose or lactate are reported as molar fractions of m0, m1, m2, etc., according to the number of labeled carbons in the molecule (13). The sum of all isotopomers of the molecules, $\Sigma m_n$, for $I = 1$ to $n$ (i.e., 3, 5, or 6 for lactate, ribose, and glucose, respectively), equal to 1 or 100. The labeled isotopomer fractions are also reported as $m_n/\Sigma m_n$, in percent, which is a distribution not affected by the dilution with unlabeled compounds. The enrichment, $\Delta \Sigma m$, is the weighted sum of the labeled species ($\Sigma m_n$ = m1 × 1 + m2 × 2 + m3 × 3...). The relative molar enrichment equals enrichment in the product, which is divided by the enrichment in the infused precursor. In glucose, $\Delta \Sigma m$ (molar fraction) + m2 + m3 = average 13C/molecule) = 3 for [1,2,3-13C3]glucose. The relative enrichment is equivalent to relative molar specific activity, the ratio of 13C activity in a product to the 13C specific activity of the infused glycerol.

**Data Interpretation—Glucose** has been considered to be a dimer resulting from the condensation of GAP and DHAP molecules. When the rate of appearance of glucose is much slower than the rate of consumption of these triose-P molecules, the labeling pattern of the top half (C-1—C-3) of the glucose molecule is expected to be the same as that of the bottom half (C-4—C-6). Asymmetry in the glucose labeling pattern can result when the two triose-P molecules are produced or removed at unequal rates as demonstrated by Previs et al. (4). Asymmetry of labeling of the top and bottom half of the glucose molecule occurs as a result of the loss of [13C3]carbon via the pentose cycle. As shown in Fig. 2, if only the sole action of the pentose cycle is considered, a loss in the M3 isotopomers by EI, due to cleaving off glucose-6-phosphate dehydrogenase via the oxidative limb of the pentose cycle producing M2 and M1 glucose. 13C(Carbon in the lower half of the glucose molecule that cycles through the non-oxidative limb of the glucose cycle (glucose-6-phosphate → ribose/xylulose phosphates → glycerolaldehyde 3-phosphate + fructose 6-phosphate) remains intact. Asymmetry in glucose labeling pattern reflecting the sole action of the pentose cycle can be demonstrated by comparing the distribution of isotopomers of the C-1—C-4 fragment (by EI) to that of the C-1—C-6 fragment (by CI).

As shown in Fig. 2, if only the sole action of the pentose cycle is considered, a loss in the M3 isotopomers by EI, due to cleaving off carbons 5 and 6 of glucose, results in a gain of M1. However, some randomization of top and bottom of the glucose molecule does occur, secondary to the action of aldolase on fructose-1,6-P, in which case proportionate amount of M3 and M2 should be lost, if the label in M3, M2, and M1 glucose is symmetrically distributed (see “Results”).

**RESULTS**

**Distribution of Lactate Isotopomers**—In order to minimize the effect of the tricarboxylic acid cycle on the final isotopomer distribution in glucose and ribose, unlabeled gluconeogenic pre-
the molecules contains 3 $^{13}$C carbon atoms in the C-1—C-4 and carbon 6 of glucose were cleaved by EI. Only 1.7–1.8% of the

Average (Ave.) and S.D. of triplicates are provided.

|        | m0  | m1  | m2  | m3  | $\Sigma m$ |
|--------|-----|-----|-----|-----|-----------|
| No insulin Ave | 0.968 | 0.003 | 0.005 | 0.024 | 0.085 |
| S.D.       | 0.002 | 0.001 | 0.001 | 0.002 | 0.005 |
| Insulin Ave | 0.954 | 0.008 | 0.007 | 0.030 | 0.112 |
| S.D.       | 0.006 | 0.001 | 0.001 | 0.003 | 0.014 |

cursors, pyruvate and glutamine, were added to the incubation media to a final concentration of 1 mM. This had the effect of diluting any $^{13}$C label coming from the randomization of the $^{13}$C label through tricarboxylic acid cycle. Under such conditions, mass isotopomer distribution in lactate is the result of conversion of glycerol to lactate diluted by unlabeled lactate from other sources. Table I shows that lactate produced from glycerol by cultured Fao cells is greatly diluted by lactate produced from pyruvate or glutamate. This observation suggests that very little $^{13}$C-labeled glycerol went into the tricarboxylic acid cycle, and this was also supported by the fact that $^{13}$C isotopomer of glutamate was not detectable in medium glutamate (data not shown). Therefore our experimental conditions allowed us to focus on the interactions of glycolytic/glucogenic, glycogen, and pentose phosphate pathways in this Faö model.

**Effect of Insulin on Glucose Release**—The release of glucose into the medium was quantitated using the medium xylitol as a recovery standard. Xylitol is co-isolated with glucose and is converted to penta-acetate during the derivatization of glucose. The derivative of xylitol shares a common C-3—C-6 fragment (at $m/z$ 187) with the glucose aldonitrile penta-acetate derivative but has a different retention time than that of the glucose derivative (Fig. 3). The inhibition of glucose release into the medium by insulin is clearly demonstrated by the reduction of the glucose peak. The inhibition can be quantitated by examining the GC ratio of glucose to that of xylitol using a standard curve. Results are shown in Fig. 4. The release of glucose into the medium was reduced from 15.1 $\mu$mol/24 h with no insulin to 4.2 $\mu$mol of glucose with insulin (Fig. 4).

**Distribution of Glucose Isotopomers**—The mass isotopomer distribution in medium glucose (new glucose) is shown in Table II. The enrichment in glucose was 0.42 and 0.44 in the insulin-treated and untreated cells, being about four times that of the glutamate (data not shown). Therefore our experimental conditions allowed us to focus on the interactions of glycolytic/glucogenic, glycogen, and pentose phosphate pathways in this Faö model.

![Figure 3](image325x402to537x729)

**FIG. 3.** Total ion chromatogram of xylitol and glucose in the incubation media. The spectra of xylitol penta-acetate and of glucose acetonitrile penta-acetate were determined by EI. Integrated, they form the chromatographic peaks for the situation of incubation in DMEM, no glucose, 10 $^{-6}$ M dexamethasone, 2 mM xylitol, 1 mM pyruvate, without insulin (top, A) or with insulin (bottom, B). Diminution of the glucose peak where insulin is present indicates inhibition of gluconeogenesis by insulin.

![Figure 4](image348x348)

**FIG. 4.** Ratio of the integrated EI spectra of the glucose and xylitol peaks at $m/z$ 187 (see Fig. 3 for details). The height of the peaks in Fig. 3 is proportional to the concentration of glucose or xylitol in the media. The xylitol peak of the integrated EI spectra at $m/z$ 187, shown in Fig. 3, was taken to be an internal standard for this calculation, as we have found there is no change in the xylitol concentration during the experiment for the two conditions (with and without insulin).

Table I: Mass isotopomer distribution in media lactate:

|        | m0  | m1  | m2  | m3  | $\Sigma m$ |
|--------|-----|-----|-----|-----|-----------|
| No insulin Ave | 0.968 | 0.003 | 0.005 | 0.024 | 0.085 |
| S.D.       | 0.002 | 0.001 | 0.001 | 0.002 | 0.005 |
| Insulin Ave | 0.954 | 0.008 | 0.007 | 0.030 | 0.112 |
| S.D.       | 0.006 | 0.001 | 0.001 | 0.003 | 0.014 |

of the glucose molecule does occur, secondary to the action of aldolase on fructose-1,6-P (18). If the label in M3, M2, and M1 glucose is symmetrically distributed, M1 can be in any of the C-1—C-4 fragment determined by EI, it can be estimated that the glucose molecule was asymmetrically labeled with 20% of M3 labeling C-1—C-3 and 80% labeling C-4—C-6. The molar fractions of M2 glucose (C-1—C-6) in Table II were comparable to the M2 of the C-1—C-4 glucose fragment (Table II, bottom). The distribution of glucose mass isotopomers deduced from the difference between isotopomers in C-1—C-6 and C-1—C-4 fragment is shown in Fig. 5. Some randomization of top and bottom
Mass isotopomer distribution in C-1–C-6 fragment of media glucose (glucose formed during experiment)

2 mM xylitol and 1.5 mM [U-13C]glycerol present with no cold glucose added. Average (Ave) and S.D. of triplicates are provided. Media glucose from insulin-treated cells was too low for this determination.

| m0 | m1 | m2 | m3 | m4 | m5 | m6 | \( \Sigma m_n \) |
|----|----|----|----|----|----|----|-------------|
| No insulin Ave ± S.D. | 0.801 | 0.071 | 0.027 | 0.092 | 0.005 | 0.002 | 0.002 | 0.444 |
| Insulin Ave ± S.D. | 0.020 | 0.011 | 0.002 | 0.006 | 0.001 | 0.002 | 0.002 | 0.037 |
| Mass isotopomer distribution in C-1–C-4 fragment of media glucose | 0.812 | 0.066 | 0.028 | 0.085 | 0.005 | 0.002 | 0.002 | 0.420 |
| No insulin Ave ± S.D. | 0.007 | 0.002 | 0.002 | 0.005 | 0.001 | 0.003 | 0.002 | 0.023 |

Asymmetric Labeling of Glucose from [13C]Glycerol in GNG

Table II

Distribution of Pentose/Ribose Isotopomers—Table IV compares the relative enrichments in ribose derived from Fao cells incubated in the presence of 12.5 mM glucose ± 10−8 M insulin to Fao cells incubated in 2 mM xylitol ± 10−8 M insulin with no cold glucose added. Again, as in the enrichment of glycogen, enrichment in ribose of the xylitol-treated cells was significantly lower than that of the glucose-treated cells. This indicates that xylitol from the medium is phosphorylated to xylulose-5-P which equilibrates with the intracellular ribose pool, diluting the labeled ribose originated from the [U-13C]glycerol. Since labeled glycerol can be incorporated into M3 ribose by the transketolase/transaldolase reaction or recycled from glucose labeled in the C-4–C-6 positions, M3 ribose was labeled only in positions C-3–C-5, which was confirmed by the loss of M3 in the C-1–C-4 fragment of ribose (data not shown). Since 13C atoms are conserved by the TK/TA reactions, the existence of M1 and M2 ribose in relatively high amounts can only be the result of G6PDH oxidation of the [1,2,3-13C]glucose and subsequent cycling via the non-oxidative pathways.

**DISCUSSION**

The loss of [13C]glycerol carbon during gluconeogenesis has been reported previously (4). When rat liver was perfused with 0.1–1.5 mM [U-13C]glycerol, glucose isotopomers with 1–6 [13C]carbon substitutions were obtained. The symmetry of labeling in the glucose molecule was not determined. However, the enrichment and pattern of these mass isotopomers were different from those of PEP or of triose-P and were not compatible with the synthesis of glucose from a simple combination of these 3-carbon precursors. Such a discrepancy was attributed to compartmentalization or heterogeneity or isotope gradient of these precursor pools. In a later study, the loss of [13C]glycerol carbon during gluconeogenesis was again observed in isolated hepatocytes (5). When hepatocytes were incubated with [3-13C]lactate/pyruvate mixture in the presence of unlabeled glycerol, substantially less 13C was found in the C-1–C-3 fragment of glucose as compared with the C-4–C-6 fragment. The loss of [13C]carbon from the C-1–C-3 position of glucose was also observed, but to a lesser degree with [2-13C]glycerol incubation (Fig. 5 of Ref. 5). Since these isotopes ([2-13C]glycerol and [3-13C]lactate/pyruvate) become the inner carbons of oxaloacetate, these labels are lost at the same rate via the tricarboxylic acid cycle. Therefore, the excessive loss of [13C]carbon from C-1–C-3 of glucose labeling with [3-13C]lactate/pyruvate or [2-13C]glycerol cannot be ex-
plained by the loss of \(^{13}\)C via the tricarboxylic acid cycle. Neither can it be explained by the lack of equilibrium in triose-P isomerase. That label in the C-1 and C-2 position of glucose is lost to a different degree is compatible with the action of G6PDH and cycling between hexose-P and pentose-P. In the present study, we observed loss of \(^{13}\)C(glycerol carbon mainly from C-1—C-3 of the glucose molecules, a pattern consistent with the loss of labeled carbon via the pentose cycle. The dilution of label in glucose by xylitol is further evidence for the participation of the pentose pathway in gluconeogenesis. In light of such evidence, gluconeogenesis is not only the result of the condensation of two triose-P molecules but also the result of the pentose cycle depending on substrate availability. Under conditions where the pentose cycle is active, glucose molecule is not just a dimer of triose-P molecules, and the application of MIDA in the study of gluconeogenesis assuming dimerization of triose-P is clearly problematic.

The underestimation of gluconeogenesis by MIDA has been an enigma. It has been mathematically proven that the approach tolerates a wide range of disequilibrium between GAP and DHAP (1). A 50% asymmetry between label in C-1—C-3 and C-4—C-6 of glucose results in less than 5% underestimation of precursor enrichment. The underestimation of precursor enrichment can only falsely increase the estimation of GNG, a condition that may be difficult to detect. Therefore, asymmetry of labeling is not the reason for underestimation of GNG. The MIDA method is, however, modestly sensitive to isotope gradient. By using the model of reference (Fig. 5 in Ref. 4), one can show that a gradient of 25% in glycerol enrichment (from 0.2 to 0.15) results in 2% underestimation of GNG. Substantial underestimation of GNG occurs when the gradient is 75%. The effect of cycling of glucose carbon through the PPP has not been studied. An example of such an effect is provided under the “Appendix.” There are three major variables that can influence the distribution of mass isotopomers in glucose. These are the oxidation of glucose-6-P by G6PDH, the dilution of glucose carbon by unlabeled pentose carbon, and finally, the substrate flux via the non-oxidative branch of the PPP relative to that of gluconeogenic flux. These three processes all have the effect of altering the mass isotopomer distribution after glucose is synthesized (by dimerization of triose-P) and can contribute to the underestimation of gluconeogenesis.

The Fao hepatoma cell is an 8-azaguanine-resistant, ouabain-resistant clone derived from the Reuber H35 rat hepatoma cell line (6, 7). These Fao cells show stable expression of a number of liver-specific functions. These include the following: 1) the expression of the gluconeogenic enzymes PEP carboxykinase and fructose-1,6-bisphosphatase (7, 15), and 2) the ability to grow in the absence of glucose and to release glucose into the medium. In addition, Fao cells have been found to have specific high affinity binding of insulin at physiologic concentrations and to respond to insulin and dexamethasone similar to other primary hepatocytes (16, 17). In the present study, the effect of insulin on the inhibition of gluconeogenesis was demonstrated. Since insulin treatment did not alter the enrichment or labeling pattern in glucose, the effect of insulin is probably to decrease the activity of glucose-6-phosphatase. The Fao cells
Asymmetric Labeling of Glucose from $[^{13}C]$Glycerol in GNG

TABLE VI
Equilibrium distribution of mass isotopomers in sedoheptulose and erythrose-4-phosphate by the action of transketolase/transaldolase

| Glucose isotopomers | Final glucose isotopomers |
|---------------------|---------------------------|
| From triose-P | From pentose cycle | 30% PC | 60% PC |
| M6 | 0.25 | 0.125 | 0.2125 | 0.175 |
| M5 | 0.125 | 0.0375 | 0.075 | 0.0375 |
| M4 | 0.125 | 0.0375 | 0.075 | 0.0375 |
| M3 | 0.125 | 0.0375 | 0.075 | 0.0375 |
| M2 | 0.125 | 0.0375 | 0.075 | 0.0375 |
| M1 | 0.125 | 0.0375 | 0.075 | 0.0375 |
| M0 | 0.25 | 0.125 | 0.2125 | 0.175 |
| m3t/m3b (asymmetry) | 19/0.23 | 18/0.33 | 0.5 | 0.5 |
| GNG | 0.85 | 0.70 |

*30% PC means 30% of the gluconeogenic flux passes through the pentose phosphate pathways.
*This is calculated by 30% $\times 0.125$ (from column 2) plus 70% $\times 0.25$ (from column 1). This method of calculation is applied to the other mass isotopomers.
*Asymmetry is calculated using information in Table VII for the m3t/m3b ratio of glucose from the pentose pathway and m3t/m3b of glucose from Table V.
*P is calculated from M6/M3 of the final glucose isomer distribution. P = $2 \times M6/M3$/(1 + 2 $\times M6/M3$).
*GNG or FSR is M3/2 $\times P \times (1 - P)$.

are known to lack glucokinase (14). We did not find any glucose uptake even in the presence of 12.5 mM glucose in the medium. We demonstrate for the first time a very active pentose phosphate cycle in these cells which is an integral part of substrate flux in gluconeogenesis.

Traditionally, the pentose phosphate cycle has been considered to be relatively unimportant in hepatic glucose metabolism. This conclusion is based on the following: 1) that the flux through the oxidative branch of the pentose cycle accounts for a small part (less than 10%) of the glucose uptake, and 2) that the quantity of pentose produced by the action of G6PDH exceeds the net synthesis of ribose in RNA. Combining data from [1,14C]-, [2,14C]-, and [6-14C]glucose studies on the pentose cycle, Katz and Rognstad (18) showed that the flux of hexose-P through the non-oxidative branch could be substantial equaling 2–3 times that of the glucose uptake. This was confirmed in a recent study using [1,2-13C2]glucose (19). We show in the present study that during gluconeogenesis, the flux through the pentose cycle could be equally substantial. The enrichment of M1 and M2 isotopomers suggests the flux to be 2–3 times that of the gluconeogenic flux. In addition to the production of xylulose-5-P and its action on the bifunctional enzyme, we believe that the pentose cycle may play other roles in the regulation of glycolysis/gluconeogenesis. The role of the pentose cycle in the regulation of hepatic glucose metabolism therefore deserve further study.

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APPENDIX

Although hexose-P flux through the oxidative branch of the pentose pathway represents only a small portion of the glycolytic/gluconeogenic flux, hexose-P flux through the non-oxidative branch has been shown to be substantial, equaling to 2–3 times that of the glucose uptake (18).

In addition, Crawford and Blum (20) showed that the magnitude of the bidirectional flux through transketolase or transaldolase exceeds the flux through the limbs of the key substrate cycles of glycolysis/gluconeogenesis (glucokinase/glucose-6-phosphatase, 6-phosphofructokinase/fructose-1,6-bisphosphatase, and pyruvate kinase/phosphoenolpyruvate carboxykinase) and glycogen storage by 2–10-fold in fed hepatocytes.

The operation of the pentose pathway therefore can substantially influence the mass isotopomer distribution in glucose and on the calculation of gluconeogenesis based on the enrichment of $^{13}$C-labeled precursors. There are three major variables that can influence the distribution of mass isotopomer in glucose. These are the oxidation of glucose-6-P by G6PDH, the dilution of glucose carbon by unlabeled pentose carbon, and the substrate flux via the non-oxidative branch of the PPP relative to that of gluconeogenic flux. In this example, we follow glucose isotopomers formed from the combination of 50% enriched [U-13C2]triose-P through the textbook account of the pentose pathway assuming no tracer dilution by unlabeled pentose (Table V). From the combination of two triose-P, we have four distinct mass and positional isotopomers, which are converted to their respective pentose phosphate counterparts. By inspection, carbons of C-1—C-2 position in the pentose phosphate are 50% $^{13}$C and 50% $^{12}$C, or 50% m2 and 50% m0. At equilibrium, there are eight isotopomers of sedoheptulose-7-P and five isotopomers of erythrose-4-phosphate (Table VI). The distribution of these isotopomers can be calculated by their respective combinatorial probabilities. For example [U-13C2]sedoheptulose-7-P is the result of combining m2 in C-1—C-2 position with m5 in the pentose-5-P, and the probability is the product of 50 and 25% giving 12.5%.

through the pentose cycle could be equally substantial. The enrichment of M1 and M2 isotopomers suggests the flux to be 2–3 times that of the gluconeogenic flux. In addition to the production of xylulose-5-P and its action on the bifunctional enzyme, we believe that the pentose cycle may play other roles in the regulation of glycolysis/gluconeogenesis. The role of the pentose cycle in the regulation of hepatic glucose metabolism therefore deserves further study.

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The labeled sedoheptulose-7-P and erythrose-4-P eventually return to fructose-6-P by either combining the top three carbons of sedoheptulose-7-P with glyceraldehyde phosphate or condensing the top 2 carbons of pentose-5-P with erythrose-4-P. The resulting distribution is again dictated by combinatorial probability (Table VII). The example is simplified by the fact that both the enrichment in glyceraldehyde phosphate (m3b) and C-1—C-2 of pentose phosphate (m2t) happen to be 50%. To facilitate the calculation of distribution from combinatorial probability using information of Table VI, we designate the origin of the precursor either as labeled top or labeled bottom (m3t, m3b, etc.). Thus, m6 glucose can be derived from combining m3t of sedoheptulose-7-P and m3b of glyceraldehyde-3-P or m2t from xylulose-5-P and m4b of erythrose-4-P. m3t in sedoheptulose-7-P is 25% and m3b in glyceraldehyde-3-P is 50%. Therefore, the probability of m6 (m3t/m3b) is 12.5% or (0.125).

The isotopomer distribution in glucose after passing through the pentose cycle is the average of those from both of these two transketolase reactions and is presented in column 2 in Table VIII.

The final mass isotopomer distribution in glucose can be considered to be the result of mixing glucose from the combination of two triose-P with the glucose recycled from the pentose cycle. The final mass isotopomer distribution depends on the relative contribution of these two pools. We consider two possibilities, one having 30% gluconeogenic flux to pass through the pentose cycle (30%) and the other 60%, shown in the 3rd and 4th columns of Table VIII. The assumption in these calculations of 30–60% of gluconeogenic flux transversing the pentose cycle may be conservative, considering the estimations of bidirectional transketolase and transaldolase flux by Crawford and Blum (20). The mass isotopomers in column 3 are the result of adding 30% of column 2 to 70% of column 1; and the isotopomers in column 4 are the result of adding 60% of column 2 to 40% of column 1. When the precursor enrichment (p) and the fractional gluconeogenesis (GNG) are calculated using MIDA, we confirm the minimal influence of the asymmetry on the calculation of p. However, substantial underestimation of GNG occurs because of the effect of the pentose cycle. Note that the asymmetry of the labeling pattern in glucose due to the pentose cycle is readily detected as indicated by m3t/m3b ratios. The asymmetry in our experiment is 1.7:9.2 (Fig. 5), suggesting that the gluconeogenic flux passing through the pentose cycle is very large.

In this example, we assume possible tracer dilution by unlabeled pentose to be relatively small. We have not discussed the effect of isotopic equilibration between the pentose phosphate and the hexose phosphate pools by just the transketolase and transaldolase reactions without the action of G6PDH. It should be pointed out that such equilibrium reactions have the same effect in introducing asymmetry in symmetrically labeled glucose precursor, i.e. M1, M2, and M3 glucose are generated from [1,2,3-13C]glucose but not from [4,5,6-13C2]glucose. Their effects on the glucose mass isotopomer distribution and calculations of MIDA are expected to be similar. As shown in the above examples (Tables V–VIII), the calculation of GNG is subject to compensating errors of overestimation of GNG due to underestimation of p and underestimation of GNG due to scrambling of isotope. From the results of Ref. 5, we can conclude that the effect of isotope scrambling dominates even in the studies using [2,13C]glycerol causing significant underestimation of GNG.

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