We previously reported (Previs, S. F., Fernandez, C. A., Yang, D., Soloviev, M. V., David, F., and Brunengraber, H. (1995) J. Biol. Chem. 270, 19806–19815) that glucose made in isolated livers from starved rats perfused with physiological concentrations of lactate, pyruvate, and either [2-13C]- or [U-13C3]glycerol had a mass isotopomer distribution incompatible with glucose being made from a homogeneously labeled pool of triose phosphates. Similar data were obtained in live rats infused with [U-13C3]glycerol. We ascribed the labeling heterogeneity to major decreases in glycerol concentration and enrichment across the liver. We concluded that [13C]glycerol is unsuitable for tracing the contribution of gluconeogenesis to total glucose production. We now report isotopic heterogeneity of gluconeogenesis in hepatocytes, even when all cells are in contact with identical concentrations and enrichments of gluconeogenic substrates. Total rat hepatocytes were incubated with concentrations of glycerol, lactate, and pyruvate that were kept constant by substrate infusions. To modulate competition between substrates, the (glycerol)/(lactate + pyruvate) infusion ratio ranged from 0.23 to 3.60. Metabolic and isotopic steady states were achieved in all cases. The apparent contribution of gluconeogenesis to glucose production (f) was calculated from the mass isotopomer distribution of glucose. When all substrates were 13C-labeled, f was 97%, as expected in glycogen-deprived hepatocytes. As the infusion ratio ([13C]glycerol)/(lactate + pyruvate) increased, f increased from 73% to 94%. In contrast, as the infusion ratio (glycerol/[13C]lactate + [13C]pyruvate) increased, f decreased from 93% to 76%. In all cases, f increased with the rate of supply of the substrate that was labeled. Variations in f show that the 13C labeling of triose phosphates was not equal in all hepatocytes, even when exposed to the same substrate concentrations and enrichments. We also showed that zonation of glycerol kinase activity is minor in rat liver. We conclude that zonation of other processes than glycerol phosphorylation contributes to the heterogeneity of triose phosphate labeling from glycerol in rat liver.

When a polymer is synthesized from a monomeric compound labeled with a stable isotope (13C, 2H, 15N, or 18O), the mass isotopomer distribution (MID) of the polymer, measured by gas chromatography-mass spectrometry, is a unique function of the isotopic enrichment (p) of the precursor subunit (1–4). This was validated in chemical models, such as (i) glucose penta-13C acetate, made by reacting glucose with samples of [13C]acetic anhydride of varying enrichments (1), and (ii) hexamethylenetetramine made from either [13C]formaldehyde and NH4Cl, or formaldehyde and 15NH4Cl (3). The MID of the polymer can be predicted from probability analysis (1–4). Conversely, computation of the MID of the polymer yields the enrichment of the monomer.

Mass isotopomer distribution analysis (MIDA) was proposed (2) as a method for estimating the fractional synthesis rate of various biopolymers (fatty acids, cholesterol, glucose) synthesized in vivo and in vitro in the presence of 13C tracers that label the appropriate monomer (acyt-CoA or triose phosphates). Computation of the MID of the biopolymer yields both the fractional synthetic rate and the enrichment of the monomeric precursor. A variant of MIDA, isotopomer spectral analysis, was presented by Kelleher et al. (4).

The validity of MIDA and isotopomer spectral analysis requires that two parameters be constant in all cells making the polymer, namely the fractional synthetic rate and the enrichment of the monomeric precursor. When the enrichment of the precursor is not constant, computation of the MID of the polymer yields erroneous values for the enrichment of the monomeric precursor and for the fractional synthesis. In fact, the calculated fractional synthesis is not the integrated average of this parameter. This is because the precursor enrichment appears in the equations for the amount of each isotopomer as a nonlinear parameter. For a detailed discussion, see Ref. 3.

Glucose can be considered as a dimer formed from the condensation of two triose phosphate molecules. Thus, MIDA of glucose labeled from 13C-labeled precursors was proposed to estimate the contribution of gluconeogenesis (GNG) to total glucose production (f) (2, 5–8). Calculations of f would not be subject to artifacts of isotopic exchange which affects calculations of GNG from the amount of 13C tracers incorporated into glucose (9, 10). However, the main underlying assumption of MIDA is that the triose phosphate pool of all gluconeogenic cells must be at about the same enrichment. To the extent that the triose phosphates are not equally labeled, f is underestimated.

Investigations of GNG have been conducted in which MIDA was applied for determining f. Some investigators have con-
cluded that MDA does provide a reliable estimation of \( f \) during the infusion of \([^{13}C] \) glycerol (5–7) or \([^{13}C]\) lactate (5, 8, 11). However, we demonstrated previously that the use of \([^{13}C]\) glycerol and MDA is limited for estimating \( f \) (11, 12). We concluded that the major mechanisms responsible for our observations were (i) a gradient of glycerol concentration and (ii) a gradient of the \( ^{13}C \) enrichment of glycerol across the liver (11–14). Thus, most likely, triose phosphates were not equally labeled from \([^{13}C]\) glycerol in all hepatocytes. Therefore, the assumption of a triose phosphate pool of homogeneous enrichment is probably not valid for all conditions. Indeed, Péroni et al. (15) infused \([^{2-13}C]\) glycerol to 2-day-starved diabetic rats with undetectable liver glycogen and calculated an impossibly low \( f \) of 43%.

In an attempt to further test our hypothesis, i.e. that \( f \) was underestimated due to heterogeneous triose phosphate labeling induced by substrate gradients across the liver, hepatocytes from 48-h fasted rats were incubated in a medium that contained glycerol, lactate, and pyruvate. Under these conditions, GNG should account for 100% of glucose production. The substrates and \( ^{13}C \) labeling were maintained glycerol, lactate and pyruvate. Under these conditions, GNG should account for 100% of glucose production. The substrate infusion ratio glycerol/(lactate + pyruvate) was varied to simulate gluconeogenic conditions favoring either glycerol or (lactate + pyruvate). Glycerol, lactate, and pyruvate were present in all experiments; however, the source of \( ^{13}C \) was alternated in different experiments (either glycerol or lactate + pyruvate). Both singly \( ^{13}C \)- and \(^{13}C\)-labeled substrates were tested. This design guaranteed that all cells had equal access to all substrates and \( ^{13}C \) labeling. Our data show that calculations of \( f \) via MDA are limited by variations in the relative contributions of glycerol versus lactate + pyruvate to triose phosphates in gluconeogenic hepatocytes. This finding is true whether one uses singly \( ^{13}C \)- or uniformly \( ^{13}C \)-labeled tracers. In addition, we found minimal zonation of hepatic glycerokinase activity in 48-h fasted rats.

### EXPERIMENTAL PROCEDURES

#### Materials—
Chemicals, enzymes and coenzymes were purchased from Sigma-Aldrich and Boehringer Mannheim. \([U-^{13}C_3]\) Lactate (99%), \([U-^{13}C_3]\) pyruvate (99%), \([^{13}C_3]\) lactate (99%), \([^{13}C_3]\) pyruvate (99%), and \([^{2-13}C] \) glycerol (99%) were from Isotec. \([^{13}C_2]\) Glycerol (99%) was from Cambridge Isotope Laboratories.

#### Hepatocyte Isolation—
Hepatocytes were isolated from 48-h fasted male Sprague-Dawley rats (180–210 g) by perfusion with collagenase, as described by Seglen (16). Cells were counted, and viability (range 83–91%) was determined by trypan blue staining (17). Following isolation, hepatocytes were washed three times with four volumes of Krebs-Ringer bicarbonate (pH 7.4) and then kept on ice in the same buffer. Typically, hepatocytes were isolated from three rats and pooled.

#### Hepatocyte Incubations—
Hepatocytes (300 mg) suspended in 20 ml of buffer, were incubated for 45 min in 125 ml Erlenmeyer flasks, under \( 95\% \) O\(_2\) and \( 5\% \) CO\(_2\) at 37 \(^\circ\)C, with orbital shaking at 75 revolutions min\(^{-1}\). To achieve variable levels of competition between substrates for GNG, different steady state substrate concentrations were used. At 0 min, the flasks were loaded with the initial concentrations of glycerol, lactate, and pyruvate shown in Table I. The initial concentrations were maintained throughout the incubation by constantly infusing each substrate at the rates indicated. These infusion rates, determined in orientation experiments, resulted in (glycerol)/(lactate + pyruvate) infusion ratios shown in the first column of Table I. For each infusion ratio, five combinations of labeled substrates were used: (i) \([^{1,13}C] \) glycerol + \([^{1,13}C] \) lactate + \([^{13}C] \) pyruvate, (ii) \([^{1,13}C] \) glycerol + lactate + pyruvate, (iii) \([^{1,13}C] \) lactate + \([^{13}C] \) pyruvate, (iv) \([^{2,13}C] \) glycerol + lactate + pyruvate, and (v) \([^{1,13}C] \) lactate + \([^{13}C] \) pyruvate.

All labeled substrates were used at 99 atom % enrichment. For each pool of hepatocytes, one flask was incubated without substrates. To check for isotopic steady state, the incubation protocol was scaled up (3 g of cells incubated in 200 ml of buffer) so that 10 samples could be obtained during a 65-min incubation. One such incubation was conducted at each (glycerol)/(lactate + pyruvate) infusion ratio for labeling schemes (i), (ii), and (iii).

### RESULTS

Steady state concentrations of lactate, pyruvate, and glycerol were achieved under all conditions tested. As an example, Fig. 1 shows these concentrations under the conditions of row 3 of Table I. Glucose production was linear (\( r = 0.99 \); Fig. 1 and last column of Table I). In control flasks incubated without any substrates, concentrations of glycerol, lactate, pyruvate, and...
glucose were very low (bottom row of Table I). Thus, our incubation conditions allow to keep hepatocytes under physiological concentrations of substrates without having to resort to perfusion techniques (25).

Table I (right column) shows that the production of glucose, expressed as a percent of the maximum possible production from the infused substrates, increased as the substrate infusion ratio glycerol/([U-13C3]lactate + pyruvate) increased. At a low infusion ratio, when lactate/pyruvate is the dominant gluconeogenic precursor, a fraction of the substrate must be oxidized in the citric acid cycle to provide energy for glucose production (6 ATP per glucose formed from lactate). Similar partial oxidation of lactate has been reported in kidney slices synthesizing glucose (26). In contrast, when glycerol is the dominant gluconeogenic substrate, the yield of glucose production is much higher because the conversion of glycerol to glucose generates ATP (4 or 2 ATP per glucose formed from glycerol, depending on whether the conversion of α-glycerophosphate to dihydroxyacetone phosphate involves reduction of NAD+ or FAD).

Isotopic steady state of glucose labeling was reached within the first 5 min of the incubation. As an example, Fig. 2 shows parameter $f$, which is the apparent contribution of GNG to glucose production under the conditions of row 3 of Table I. Under these conditions, when all the substrates were U-13C3-labeled, $f$ was 97%, as expected in glycogen-depleted hepatocytes. However, when glycerol was U-13C3-labeled and lactate and pyruvate were unlabeled, $f$ was 93%. Additionally, when lactate and pyruvate were U-13C3-labeled and glycerol was unlabeled, $f$ was 78%. In the case when lactate and pyruvate were U-13C3-labeled, the first point, obtained at 5 min during the incubation, is slightly lower than the average of the other points (i.e. 71% versus 78%). This does not significantly impact on the calculation of $f$ from glucose obtained at 45 min during the incubation. This is because (i) the $f$ calculated at 5 min (i.e. 71%) is within 91% of the final $f$ (i.e. 78%) and (ii) the amount of glucose produced after only 5 min represents less than 10% of the total glucose produced during the incubation. Similar isotopic steady states were maintained using the other substrate infusion ratios (data not shown).

The enrichment of glycerol, lactate and pyruvate was measured in samples obtained at the end of the incubation. When glycerol and [3-13C]lactate + [3-13C]pyruvate were infused, the glycerol pool actually became 13C-labeled during the incubation. As the substrate infusion ratio (glycerol)/([3-13C]lactate + [3-13C]pyruvate) increased from 0.23 to 0.61, 1.4, and 3.6, the enrichment of glycerol at 40 min was 12 ± 3%, 5 ± 2%, 3 ± 1%, and 1 ± 1%, respectively. When [2-13C]glycerol and lactate + pyruvate were infused, labeling of the (lactate + pyruvate) pool (4 ± 2%) was observed only at the highest substrate infusion ratio ([2-13C]glycerol)/([lactate + pyruvate]) of 3.60.

Fig. 3 shows $f$ calculated in all protocols that used U-13C3-labeled substrates. When all substrates were uniformly 13C-labeled, $f$ was 97%, as expected in glycogen-deprived hepatocytes. As the infusion ratio ([U-13C3]glycerol)/([lactate + pyruvate]) increased, $f$ increased from 73% to 94%. In contrast, as the infusion ratio ([glycerol]/([U-13C3]lactate + [U-13C3]pyruvate)) increased, $f$ decreased from 93% to 76%. In all cases,

\[
\text{Table I}
\]

Rates of substrate infusion and of glucose production in hepatocyte incubations

At 0 min, the listed millimolar concentrations of glycerol, lactate, and pyruvate were added to the flasks containing hepatocytes suspended in buffer. The substrates were then infused from 0 to 45 min. Shown are the millimolar concentrations of each substrate at 40 min, the substrate infusion rates (μmol/min · g wet weight) required to maintain these concentrations, and rates of glucose production (μmol/min · g wet weight, or percent of maximum). Data are presented as mean ± S.E. The n in each group is the number of incubations in which all substrates were U-13C3-labeled. Similar data were obtained from incubations with other labeling schemes. Conc., concentration.

| Infusion ratio (glycerol)/([lactate + pyruvate]) | Substrate | Glucose production |
|-----------------------------------------------|-----------|-------------------|
|                                               | Conc. at t = 0 | Conc. at t = 40 | Rate (%) | Maximum (%) |
| 0.23 (n = 7)                                   | 0.025      | 1.0              | 0.18     | 0.61 ± 0.05 | 65 ± 5     |
|                                               | 0.025 ± 0.004 | 1.0 ± 0.04           | 0.18 ± 0.01 |        |           |
|                                               | 0.35       | 0.12             | 1.40     |        |           |
|                                               | 0.04       | 1.0              | 0.15     |        |           |
| 0.61 (n = 5)                                   | 0.04 ± 0.005 | 1.0 ± 0.05          | 0.15 ± 0.02 | 0.89 ± 0.07 | 79 ± 4     |
|                                               | 0.85       | 0.10             | 1.30     |        |           |
| 1.40 (n = 6)                                   | 0.30 ± 0.03 | 1.0 ± 0.07        | 0.10 ± 0.02 | 1.44 ± 0.08 | 86 ± 4     |
|                                               | 1.95       | 1.0              | 1.35     |        |           |
| 3.60 (n = 6)                                   | 0.25       | 1.0              | 0.06     |        |           |
| Control                                       | 1.80       | 0.05             | 0.45     |        |           |
| No substrates (n = 6)                          | 0.25 ± 0.03 | 1.0 ± 0.05        | 0.06 ± 0.02 | 1.04 ± 0.04 | 90 ± 3     |
|                                               | 1.80       | 0.05             | 0.45     |        |           |
|                                               | <0.07      | Undetectable     |          | Undetectable | NA       |
increased with the rate of supply of the substrate that was labeled.

We compared the \( f \) values calculated from U-\(^{13}\)C\(_3\)-labeled substrates with those calculated from singly \(^{13}\)C-labeled substrates. Fig. 4 shows that practically identical \( f \) were calculated from either U-\(^{13}\)C\(_3\)- or singly \(^{13}\)C-labeled substrates. The solid circles represent experiments in which \(^{13}\)C-glycerol plus unlabeled lactate and pyruvate were infused, and the open circles represent experiments in which unlabeled glycerol plus \(^{13}\)C-lactate and \(^{13}\)C-pyruvate were infused.

In contrast, as the infusion ratio (glycerol)/(lactate + pyruvate) increased, triose phosphate equilibration increased from 89 to 77%. In all cases, triose phosphate equilibration increased with the rate of supply of the substrate that was labeled.
To test the linearity of our measurements of isotopomer distributions, for each labeling pattern, we pooled final samples of incubation medium from experiments in which the (glycerol)/(lactate + pyruvate) infusion ratio was 1.4. After measurement of the MID of each pool, seven increasing dilutions of the [13C]glucose (up to 20-fold) were made by adding increasing amounts of a solution of unlabeled glucose. Then, after purification on resins, the MID of the diluted glucose samples were measured, converted to f values that were compared with f values calculated using the unlabeled glucose dilution factor. The regression slopes of the seven points of each comparison ranged from 0.965 to 0.99, with

\[ r^2 > 0.99. \]

Thus, we are confident of the precision and linearity of our measurements of the MID of glucose, whether labeled from singly or uniformly 13C-labeled substrates.

To test whether low values of f obtained in previous in vivo and perfused rat liver experiments with [13C]glycerol (11, 12) resulted from zonation of glycerol kinase, we isolated hepatocytes from the perportal and percentral areas of the liver. Table II shows, for livers of 2-day-starved rats, the total activity and zonal distribution of glutamine synthetase, lactate dehydrogenase, and glycerol kinase. Data for zonation of glutamine synthetase and lactate dehydrogenase, as well as total activity of glycerol kinase, are in agreement with published reports (27, 28). We found no sizeable zonation of glycerol kinase activity in hepatocytes (Table II).

**DISCUSSION**

MIDA was proposed by Neese et al. (5) as an effective and reliable method for estimating f, the contribution of GNG to total glucose production. Glucose can be considered as a dimer of triose phosphates, the 13C enrichment of which should be very similar under most conditions. Neese et al. showed, with mathematical simulations, that a lack of perfect equilibration between the enrichments of the triose phosphates has minor impact on calculations of f, unless the ratio of enrichments of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate is greater than 1.6 or smaller than 0.65. Thus, MIDA should be applicable to studies of GNG provided that (i) the ratio of enrichments of triose phosphates across the liver lobule is not constant. This could result from zonation of glycerol kinase or from a substantial decrease in glycerol concentration across the liver lobule. We found no data on the zonation of glycerol kinase in the literature. We showed that the concentration of glycerol decreases up to 4-fold across the liver of dogs (13), and up to 5-fold across the splanchnic bed of humans (14). In addition, we showed (i) an up to 4.5-fold decrease in glycerol enrichment across the liver of overnight-starved dogs (13), and (ii) an up to 4.5-fold decrease in glyceraldehyde enrichment across the splanchnic bed of 60-h-fasted humans (14), demonstrating the production of glycerol by the liver. The large uptake of physiological concentrations of glyceraldehyde across liver and the production of glycerol by the liver were confirmed in perfused rat liver experiments (11). Based on the above data, we concluded that [U-13C3]glycerol is not suitable for tracing GNG in vivo, but that [U-13C3]lactate might be suitable, at least under some conditions.3

In contrast to our findings, Neese et al. (5) and Péroni et al. (6, 7) reported values of f of 88% and 87% in 2-day-starved rats infused with [2-13C]glycerol. They concluded that this [2-13C]glycerol is suitable for tracing GNG in vivo. The discrepancy between our findings (11) and those of Neese et al. (5) and Péroni et al. (6, 7) may result from the fact that they infused large amounts of [2-13C]glycerol (resulting in plasma glyceraldehyde enrichment of about 50%), which presumably decreased the concentration and enrichment gradients of glycerol across the liver. Indeed, we found (11) that f increased from 75% to 92% when the concentration of glycerol in liver perfusate was increased from 0.1 to 1.5 mM, respectively.

Recently, Péroni et al. (15) traced GNG in streptozotocin-diabetic rats by MIDA of glucose labeled from [2-13C]glycerol or [3-13C]lactate. They found abnormally low values of f measured with either tracer. Thus, under conditions of intense GNG, the 13C enrichment of triose phosphates, labeled from either tracer, is not uniform across the liver lobule. This probably results from the combination of (i) a large fractional uptake of glycerol and lactate in a single pass of blood across the liver, and (ii) an intense hepatic proteolysis supplying unlabeled gluconeogenic substrates in some area of the lobule.

In the present study, we measured glycerol kinase in perportal and perivenous hepatocytes and found only a 10% decrease in activity across the lobule (Table II). Thus, we can probably exclude a zonation of glycerol kinase as an explanation for the low values of f measured with [U-13C3]glycerol. However, zonation of glycerol metabolism probably results from the decrease in glyceraldehyde concentration and/or enrichment across the lobule. Thus, the experiments reported here were performed to test whether an accurate estimation of f could be achieved when all hepatocytes are exposed to the same concentrations and enrichments of substrates. Freshly isolated rat hepatocytes were incubated with glycerol, lactate, and pyruvate. These gluconeogenic substrates were maintained at steady state concentrations by constant infusions following a loading bolus. For each of the four conditions of competition between gluconeogenic substrates (i.e. different (glycerol)/(lactate + pyruvate) infusion ratios), we tested seven labeling schemes. In addition, we tested whether singly 13C- and uniformly 13C-labeled substrates yield similar values of f. During discussions with the groups of Hellerstein and Beylot, they had raised the possibility that [U-13C3]glycerol might yield impre-

3 We found (11) similar values of f in overnight-fasted monkeys infused with [U-13C3]lactate (80%) or [3-13C3]lactate (81%).
cise calculations of $f$ because of the greater number of mass isotopomers of glucose generated from this tracer compared with [2-$^{13}$C]glycerol.

When all of the infused substrates were U-$^{13}$C$_3$-labeled, the same $f$ was calculated i.e. $\sim 97\%$, regardless of the (glycerol)/ (lactate + pyruvate) infusion ratio (Fig. 3). Control incubations, in which no substrates were added, did not produce any glucose as determined by enzymatic assay. Thus, in our experiments, hepatocytes released glucose only from GNG. Therefore, this $f$ of $97\%$ is in agreement with what we should expect in glycerol-depleted hepatocytes. Presumably, the incubations with [U-$^{13}$C$_3$]glycerol, [U-$^{13}$C$_3$]lactate, and [U-$^{13}$C$_3$]pyruvate resulted in an uniform labeling of the triose phosphates in all hepatocytes.

When only glycerol was labeled, the apparent contribution of GNG to glucose production increased as the [U-$^{13}$C$_3$]glycerol/[lactate + pyruvate] infusion ratio increased (Fig. 3). At the substrate infusion ratio of 0.23, GNG apparently accounted for $\sim 73\%$ of the glucose production. Under these conditions, the steady state concentrations of glycerol, lactate, and pyruvate are physiological, and the contribution of glycerol to total exogenous substrate metabolism is 19$\%$ (calculated from the infusion rates of the substrates, Table I). Still, although all hepatocytes were in contact with the same concentrations and enrichments of substrates, the low $f$ value of 73$\%$ reveals a lack of identity of the enrichment of triose phosphates among the cells. Only when the [U-$^{13}$C$_3$]glycerol/[lactate + pyruvate] infusion ratio was increased to an unphysiological level of 3.6, did $f$ reach a value of plausible 92$\%$, but which was still significantly different from conditions where all substrates were labeled ($p < 0.05$).

When only the lactate/pyruvate pair was labeled, the apparent contribution of GNG to glucose production decreased as the glycerol/[U-$^{13}$C$_3$]lactate + [U-$^{13}$C$_3$]pyruvate] infusion ratio increased (Fig. 3). At the physiological infusion ratio of 0.23, $f$ was a plausible 93$\%$, but which was still significantly different from conditions where all substrates were labeled ($p < 0.05$). At high glycerol/[U-$^{13}$C$_3$]lactate + [U-$^{13}$C$_3$]pyruvate] infusion ratios, $f$ decreased to implausible values (77–78$\%$).

Identical values of $f$ were obtained with singly labeled and uniformly labeled substrates at all glycerol/[lactate + pyruvate] infusion ratios (Fig. 4). This demonstrates that, as far as MIDA calculations are concerned, [U-$^{13}$C$_3$]glycerol and [2-$^{13}$C]glycerol are equivalent, despite claims to the contrary (5–7, 29). However, as we mentioned previously (11), measurements of the MID of glucose is more precise with [U-$^{13}$C$_3$]-glycerol than with [2-$^{13}$C]glycerol when low rates of tracer infusion are used, resulting in low enrichment of glucose. This is because the M3 and M6 enrichments of glucose measured with [U-$^{13}$C$_3$]glycerol involve extremely low corrections for natural enrichments. In contrast, the M1 and M2 enrichments of glucose measured with [2-$^{13}$C]glycerol involve much larger corrections for natural enrichment. Presumably, this is why Neese et al. (5) used large infusion rates of [2-$^{13}$C]glycerol, resulting in (i) unphysiological concentrations and loads of glycerol, and (ii) blunting of the concentration and enrichment gradients of glycerol across the liver.

Fig. 5 addresses the degree of isotopic equilibration of the triose phosphates at different degrees of competition between substrates that label initially dihydroxyacetone phosphate (glycerol) or glyceraldehyde 3-phosphate (lactate/pyruvate). Note that the data as plotted reflect the degree to which the initially labeled triose phosphate equilibrates with its partner (see “Calculations” and legend for Fig. 5). The degree of equilibration increases as the contribution of the labeled substrate to the total substrate supply increases. A similar lack of triose phosphate equilibration had been observed by Teng et al. (30) in liver slices from fed rats, incubated with 30 mm [2-$^{13}$C]pyruvate and 30 mm glycerol. The specific activity of dihydroxyacetone phosphate was only $\sim 60\%$ of that of glyceraldehyde 3-phosphate. This corresponds to a 67$\%$ equilibration. However, the incomplete equilibration of triose phosphate labeling we observed has minor impact on the calculation of $f$ (less than 1$\%$), as demonstrated by Neese and Hellerstein (31).

The low values of $f$ we observed do not result from zonation of glycerol kinase, differences in substrate concentrations available to cells, or from the lack of complete equilibration of the triose phosphates. The data can only be explained by differences in the relative contributions of glycerol versus lactate/pyruvate to triose phosphate formation and glucose production, resulting in different labeling of triose phosphates between cell populations. If the labeling of triose phosphates were not uniform, differences in absolute rates of glucose production between cell populations would further decrease $f$. There is evidence that the activity of phosphoenolpyruvate carboxykinase (PEPCK) is zonated with periportal/perivenous activity ratios ranging from 1.5 to 3.0 (32–35). Thus, because glycerol kinase activity is not zonated (Table II), cells of perivenous origin with high PEPCK activity, when incubated with unlabeled glycerol + [1-$^{13}$C]lactate + [1-$^{13}$C]pyruvate, would have higher enrichment in triose phosphates than cells of perivenous origin. The situation would be qualitatively reversed if these cells were incubated with [1-$^{13}$C]glycerol + unlabeled lactate/pyruvate. Because PEPCK is the main flux-controlling enzyme in GNG from lactate/pyruvate (36), it may play a key role in the heterogeneity of triose phosphate labeling in livers that metabolize (lactate + pyruvate) and glycerol. However, the zonation of other enzyme activities may also play a role.

What is the relevance of our data to measurements of GNG in vivo? First, singly and uniformly 13C-labeled substrates yield the same estimates of $f$. Second, our data show that the labeling of hepatocyte triose phosphates approaches uniformity, and $f$ approaches 100$\%$, when the labeled substrate used to trace GNG is the dominant gluconeogenic substrate. Therefore, [1-$^{13}$C]glycerol might be used to trace GNG under conditions of high glycerol supply resulting in the blunting of the portohepatic glycerol concentration and enrichment gradients, and in the flooding of the triose phosphate pool with glycerol carbon. This probably occurs during total parenteral nutrition with lipid emulsions. Third, because under physiological conditions, 80–90$\%$ of gluconeogenic substrates pass through the lactate/pyruvate pool, tracers of [1-$^{13}$C]lactate and/or [1-$^{13}$C]pyruvate are probably better suited for measuring the contribution of GNG to glucose production using MIDA. However, the recent report by Péroni et al. (15) shows that neither [1-$^{13}$C]glycerol nor [1-$^{13}$C]lactate is suitable for tracing GNG by MIDA under the conditions of intense GNG that prevail in insulin-dependent diabetes. In conclusion, MIDA of glycerol, labeled from [1-$^{13}$C]lactate or [1-$^{13}$C]pyruvate, appears valid to trace only low or moderate rates of GNG.

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Limitations of the Mass Isotopomer Distribution Analysis of Glucose to Study Gluconeogenesis: HETEROGENEITY OF GLUCOSE LABELING IN INCUBATED HEPATOCYTES

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