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# Determination of pathways of glycogen synthesis and the dilution of the three-carbon pool with [U-$^{13}$C]glucose

(gas chromatography/mass spectroscopy/mass isotopomers/tricarboxylic acid cycle/gluconeogenesis/[1-$^{14}$C]- and [$^{3}$H]glucose)

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**ABSTRACT** **Rats were infused with glucose at 30 mg/min, containing 18% enriched [U-$^{13}$C]glucose and [1-$^{14}$C]- and [3-$^{3}$H]glucose. The mass isotopomer patterns of $^{13}$C-labeled blood glucose and liver glycogen were determined by gas chromatography/mass spectroscopy. The contribution of the direct pathway to glycogen was calculated from the three tracers, and the values by all three were nearly identical, about 50%. The $^{14}$C specific activity in carbon 6 of glycogen glucose was about 6% that of carbon 1. The [$^{3}$H]glucose/[1-$^{14}$C]glucose ratio in glycogen was 80–90% that in blood glucose. The enrichment of $^{13}$C and the specific activity of $^{14}$C in glycogen formed by the indirect path were 20–25% of glycogen formed directly from glucose. The dilution is of two kinds: (*i*) an exchange of labeled carbon with unlabeled carbon in the tricarboxylic acid cycle and (*ii*) dilution by unlabeled nonglucose carbon. Methods to calculate the two types of dilution are presented. In control rats the dilution factor by exchange in the tricarboxylic acid cycle is 1.4, and the dilution by unlabeled carbon is 2.5- to 3.0-fold, with the overall dilution about 4-fold. In rats preinjected with glucagon, the dilution through the tricarboxylic acid cycle was unaffected but that by nonglucose carbon was decreased.**

It is now generally realized that only a part of liver glycogen is derived from intact glucose (1). However, there still prevails confusion as to what the direct path represents and how its contribution to glycogen may be determined. Very little attention has been paid to the indirect path, the synthesis of glycogen from three-carbon compounds. We have previously described (2, 3) the application of gas chromatography/mass spectroscopy (GC/MS) with [U-$^{13}$C]glucose to the determination of the contribution of the direct path. Herein we compare the use of [U-$^{13}$C]-, [1-$^{14}$C]-, and [3-$^{3}$H]glucose for this determination. In the present study we also examine, by using [U-$^{13}$C]glucose and GC/MS, the incorporation of carbon by the indirect path into glycogen. Pyruvate from [U-$^{13}$C]glucose is, in its conversion to phospho*enol*pyruvate (PEP), diluted by exchange of [$^{13}$C]carbon with unlabeled carbon. There is also a larger dilution of the three-carbon pool by inflow from nonglucose carbon. Methods to estimate the two kinds of dilution from the isotopomer distribution were derived.

## EXPERIMENTAL PROCEDURES

Indwelling duodenal catheters were implanted in rats. The treatment of the animals and experimental design were as described (2), except that the infusate contained 18% [U-$^{13}$C]-, [1-$^{14}$C]-, and [3-$^{3}$H]glucose. Rats were starved for 24 hr and infused with glucose at 30 mg per min per kg for 160 min, with a priming dose equal to 30 min. Three rats were injected subcutaneously with 10 $\mu$g of glucagon 20 min before infusion, and three rats were not treated. At the end of the infusion, portal and arterial blood was sampled and liver was homogenized in 6% (vol/vol) perchloric acid, and glucose and glycogen were isolated. Arterial blood glucose at sacrifice (165 min after start of infusion) averaged 6.5 $\mu$mol/g of blood, and portal blood glucose was 9.5 $\mu$mol/g of blood, but $^{13}$C enrichment and specific activities in portal and arterial blood were virtually the same. Liver glycogen content averaged 85 $\mu$mol/g. Glycogen content of fasted rats was very low, from 0.7 to 1.3 $\mu$mol/g ($n = 3$), and no correction for initial glycogen content was applied. GC/MS analysis and corrections for natural abundance and $^{12}$C in [U-$^{13}$C]glucose were as described (2–4). Spectrograms of the infusate, blood glucose, and glycogen have been published elsewhere (3).

The total infused dose of [$^{14}$C]- and [$^{3}$H]glucose was 120 × $10^6$ cpm each. Blood and glycogen glucose were degraded to obtain the specific activity of $^{14}$C in C-1 and C-6 of glucose. C-1 of glucose was released enzymatically as $CO_2$. The incubation mixture was 0.1 M Hepes·NaOH (pH 8), 8 mM NADP, 5 mM ATP, 10 mM $MgCl_2$, hexokinase (5 units/ml), glucose-6-phosphate dehydrogenase (10 units/ml), and 0.6 unit of phosphogluconate dehydrogenase. Total volume was 5 ml containing 10 $\mu$mol of glucose. Incubation was overnight at room temperature. $CO_2$ was released by acidification with $H_2SO_4$ and was trapped in 2 ml of 50% (wt/vol) phenethylamine. The activity of C-6 of glucose after periodate oxidation was obtained as the dimedon derivative of formaldehyde as described by Bloom (5).

**Definitions.** In this paper $m_i$ and $M_i$ refer to the molar fractions of glucose isotopomers containing no $^{13}$C ($m_0$ and $M_0$) or containing from one to six labeled carbons (corrected for natural abundance); $M$ and $m$ refer to the mass isotopomers in blood glucose and glycogen glucose, respectively. When there is no ambiguity, the subscripts in the terms are omitted for simplicity. To illustrate, $\Sigma_1^6 m = m_1 + m_2 + m_3 + \ldots + m_6$ is the sum of the molar fractions containing $^{13}$C with masses from 181 to 186, expressed as percent, and $m_0 + \Sigma_1^6 m = 100\%$.

The number of $^{13}$C carbons in the $m_i$ fraction is designated as $n_i$. Thus, $\Sigma_1^6 mn = m_1 + m_2 \times 2 + m_3 \times 3 + \ldots + m_6 \times 6$, and this equals the molar enrichment of $^{13}$C in glucose expressed as percent. (The molar enrichment in infused 100% [U-$^{13}$C]glucose is 600%.) The conventional term for enrichment is atom percent excess (APE), equal to $\Sigma_1^6 mn/6$ for glucose, a term not used by us. The term relative molar enrichment (or relative enrichment, for short) used here is exactly equivalent to the relative molar specific activity with radioactive tracers.

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Abbreviations: GC/MS, gas chromatography/mass spectroscopy; PEP, phospho*enol*pyruvate; TCA, tricarboxylic acid.

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**Pathways of Glycogen Synthesis.** Fig. 1 represents a simplified schematic model of glycogen synthesis from [U-$^{13}$C]glucose by the two pathways. Blood glucose is assumed to be enriched 50% in [U-$^{13}$C]glucose, and the direct path is assumed to contribute 40% to glycogen. The concentration of [U-$^{13}$C]glucose in glycogen is 20%, and thus the direct path is 20/50 = 40%, and the indirect path is 60%.

It is apparent that the direct path represents the fraction of glycogen derived from intact glucose. This definition applies to all types of tracers, whether glucose is labeled with $^{13}$C, $^{14}$C, or $^{3}$H. As was stressed elsewhere (6), contribution of the pathways does not provide a measure of enzyme rates nor of the net uptake of glucose by liver.

In Fig. 1, in the indirect path the pyruvate formed by glycolysis from glucose uniformly contains $^{13}$C in all its carbons ($m_3$). It is shown to be diluted by unlabeled pyruvate from nonglucose carbon. In the conversion of pyruvate to oxaloacetate and PEP, some of the $^{13}$C is exchanged with unlabeled carbon from acetyl-CoA via the tricarboxylic acid (TCA) cycle. The extent of exchange depends on the parameters of the TCA cycle and the input of labeled acetyl-CoA derived with pyruvate dehydrogenase. PEP is composed of a variable amount of molecules containing three, two, one, or no [$^{13}$C]carbons. There also may be dilution by unlabeled carbon (from serine, glycerol, and others; data not shown) in the conversion of PEP to triose phosphates. The triose phosphates are converted to hexose phosphates and ultimately to glycogen. It should be noted that in the conversion

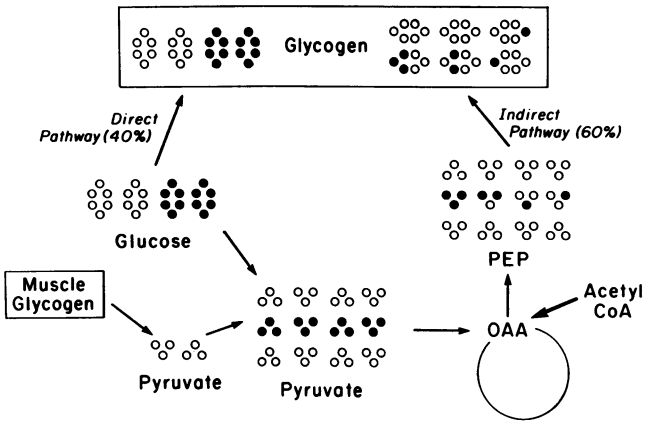


FIG. 1. Simplified scheme depicts the synthesis of glycogen from glucose labeled uniformly with $^{13}$C, 40% by the direct path and 60% by the indirect path. The six carbons of [$^{12}$C]glucose are shown as open circles, and the six carbons of [$^{13}$C]glucose are shown as solid circles. The glucose is assumed to be made 50% of [$^{12}$C]- and 50% of [$^{13}$C]carbons. The glycogen formed by the direct path contains the same concentration of unchanged [$^{13}$C]glucose as that in blood (50%). In glycolysis glucose is cleaved to pyruvate. The pyruvate contains 50% of molecules labeled uniformly with $^{13}$C. It is assumed that the pyruvate pool is diluted by one-half with unlabeled pyruvate from muscle glycolysis, so that the concentration of labeled pyruvate is decreased to 33.3%. Pyruvate is converted to PEP without a net loss of carbon, but labeled carbon is exchanged in the operation of the TCA cycle with unlabeled carbon. The labeled PEP consists of a mixture of molecules containing one, two, or three carbons. The composition depends on the parameters of the TCA cycle and input of $^{13}$C from acetyl-CoA. In the example shown here, 12 molecules of PEP containing 4 labeled molecules are converted to 6 molecules of hexose 6-phosphate and glycogen, which will contain 4 labeled molecules with $^{13}$C in either C-1, -2, and -3 or C-4, -5, and -6 of glucose. [Recombination to yield glucose containing $^{13}$C in both "top" and "bottom" is neglected in the scheme. Recombination is negligible if the concentration of labeled molecules is low (see text).] It is apparent that the contribution of the direct path is provided by the concentration of uniformly labeled molecules in glycogen, as compared to that in blood glucose (20/50 × 100 = 40%). OAA, oxaloacetate.

of $P$ mol of triose phosphate containing $Q$ labeled molecules, $P/2$ mol of hexose phosphates are formed that, however, contain $Q$ labeled molecules. In Fig. 1, the $^{13}$C in glycogen derived by the indirect path is diluted by two types of reactions—by an exchange of $^{13}$C with unlabeled carbon and by the influx of unlabeled carbon from nonglucose sources. We designate the dilution by exchange in the TCA cycle by $E$ and that by nonglucose carbon by $F$, and total dilution is thus $E \times F$. The direct path and both types of dilution may be calculated from the mass isotopomer distribution in blood glucose and glycogen.

**Calculations.** The contribution of the direct path $D$ was calculated when [1-$^{14}$C]glucose was infused essentially according to Shulman (7, 8).§

$$D = \frac{[(\text{C1} - \text{C6}) \text{ in glycogen glucose}]}{[(\text{C1} - \text{C6}) \text{ in blood glucose}]}, \quad [1]$$

where C1 and C6 refer to specific activities of C-1 and C-6 in glucose.

The direct path as calculated from [3-$^{3}$H]glucose equals simply the ratio of specific activities of glycogen and blood glucose. Equations to calculate the parameters of glycogen synthesis and dilutions from the mass isotopomer distribution in glucose and glycogen are presented in the *Appendix*. Eq. 2 represents the relative enrichment, the ratio of $^{13}$C in glycogen to that in blood glucose. This expression is exactly equivalent to the relative specific activity from [U-$^{14}$C]glucose. The simple expression $D = m_6/M_6$ (Eq. 3), the ratio of the concentration of glycogen of mass $m_6$ to that in blood glucose ($M_6$), yields the contribution of the direct path. Eq. 3 is valid even if blood glucose contains labeled species other than $M_6$ formed due to recycling (see below). The reason is that as shown here the recombination of triose phosphates to form labeled $M_6$ is negligible. Thus, Eq. 3 would be valid if, for example, a mixture of [1-$^{13}$C]- and [U-$^{13}$C]glucose were infused. The derivations of Eqs. 4 and 5, the dilution of carbon in glycogen by way of the indirect path, are presented in the *Appendix*.

## RESULTS

**Direct Path.** In Table 1 we present the mass isotopomer distribution in arterial and glycogen glucose from experiments with rats infused with a mixture of 18% [U-$^{13}$C]-, [1-$^{14}$C]-, and [3-$^{3}$H]glucose as well as the ratios of enrichment and specific activity of glycogen to that in blood glucose. The relative specific activity of $^{14}$C- or enrichment of $^{13}$C-labeled glycogen provide the possible upper limit for the contribution of the direct path.

In Table 2, the contribution of the direct path as calculated from [U-$^{13}$C]-, [1-$^{14}$C]-, and [3-$^{3}$H]glucose is presented. The agreement by the three methods is very close. With untreated rats the contribution of the direct path of 50% is in good agreement with previous studies (1). The values are somewhat higher than the results of Shulman *et al.* (7–9) of 35–45% with similar glucose loads. The contribution of the direct path in glucagon-treated rats was higher, 60–70%, but the number of animals is rather limited.

Table 2 presents also the incorporation of $^{14}$C into C-6 of glycogen from [1-$^{14}$C]glucose. The C6/C1 activity ratios of 0.06 to 0.07 are quite similar to the randomization of [1-$^{13}$C]glucose in glycogen, as determined with NMR by

§In primed intragastric infusion, the enrichment and specific activity in portal blood and liver exceeded initially that in peripheral blood. It remained higher than in arterial or venous blood until steady state was attained, between 60 and 90 min. Thus, a correction for the period before steady state was attained in venous blood was not appropriate, and none was applied.

Table 1. Mass isotopomer distribution and relative specific activity in glycogen

| Exp. | Pretreatment | Molecule | Mass isotopomer distribution,* % | | | | | | Tracer in glycogen†/ tracer in glucose | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $m_0$ | $m_1$ | $m_2$ | $m_3$ | $m_{4,5}$ | $m_6$ | $^{13}$CU | $^{14}$C1 | $^{3}$H3 |
| 1 | None | Glucose | 81.0 | 1.2 | 1.0 | 1.1 | 0.5 | 15.0 | 0.65 | 0.58 | 0.51 |
| | | Glycogen | 84.7 | 1.7 | 2.1 | 2.7 | 0.8 | 7.8 | | | |
| 2 | None | Glucose | 81.4 | 1.0 | 0.6 | 0.8 | 0.5 | 15.6 | 0.68 | 0.60 | 0.51 |
| | | Glycogen | 82.6 | 2.8 | 2.7 | 3.2 | 0.9 | 7.8 | | | |
| 3 | Glucagon‡ | Glucose | 81.2 | 0.8 | 0.5 | 0.7 | 0.1 | 16.6 | 0.85 | 0.86 | 0.73 |
| | | Glycogen | 80.1 | 2.0 | 2.0 | 3.6 | 0.5 | 11.5 | | | |
| 4 | Glucagon‡ | Glucose | 80.7 | 1.3 | 1.1 | 1.3 | 0.6 | 14.8 | 0.78 | 0.76 | 0.69 |
| | | Glycogen | 81.1 | 2.4 | 2.7 | 3.7 | 1.0 | 8.9 | | | |

Rats were infused with a mixture of 18% enriched [U-$^{13}$C]-, [1-$^{14}$C]-, and [3-$^{3}$H]glucose. $^{13}$CU, $^{14}$C1, $^{3}$H3, activities of uniformly labeled glucose and of glucose labeled at C-1 and H-3, respectively.
*Corrected for natural abundance of $^{13}$C and $^{12}$C in the infused glucose.
†Enrichment of [U-$^{13}$C]-, [1-$^{14}$C]-, and [3-$^{3}$H]glucose specific activities of glycogen relative to enrichment and specific activities in blood glucose.
‡Injected i.p. 20 min before infusion with 20 $\mu$g of glucagon.

Shulman *et al.* (8, 9). If there was no dilution of the pyruvate from [1-$^{14}$C]glucose, the C6/C1 ratio for a 50% direct path should have been 0.33.

Table 2 presents also the [3-$^{3}$H]glucose/[1-$^{14}$C]glucose ratios in glycogen. They ranged from 0.8 to 0.9. Most if not all of the $^{3}$H of glucose is lost in its conversion to pyruvate and its recycling to glucose. Hence, with [$^{3}$H]glucose, the incorporation of $^{3}$H into glycogen occurs virtually by the direct path. Thus, with [3-$^{3}$H]- or [6-$^{3}$H]glucose, the relative specific activity in glycogen provides a simple estimate of the direct path. This is illustrated by comparing the specific activities (Table 1) and the contribution of the direct path (Table 2). The $^{3}$H/$^{14}$C ratio depends largely on the dilution of labeled carbon in the glycogen fraction formed by the indirect path. The high $^{3}$H/$^{14}$C ratio has been used erroneously by Huang and Veech (10) and in other studies as evidence for a negligible contribution of an indirect path.

**Indirect Path.** Table 3 provides a comparison of the direct path with the relative enrichment and relative specific activity of glycogen from [U-$^{13}$C]- and [1-$^{14}$C]glucose. The relative enrichment and specific activity are slightly higher than the direct path, indicating a low content of labeled carbon in the indirectly formed glycogen. For the average of eight experiments, the enrichment of $^{13}$C in the fraction from three-carbon compounds is about (59 − 49)/49 or 20% of that formed directly from glucose. The low tracer content of the former fraction is also apparent from the low C6/C1 and high $^{3}$H/$^{14}$C activity ratios shown in Table 2.

The mass isotopomer data permit a more exact and separate estimate of dilution by exchange of $^{13}$C with unlabeled carbon, $E$, and by nonglucose carbon, $F$, and of total dilution, $E \times F$. The dilution by exchange is remarkably uniform, ranging from 1.3 to 1.5. The dilution by nonglucose in untreated rats ranged from 2.1 to 3.7, averaging 2.7, and total dilution was close to 4. There are only three experiments with glucogen-treated rats that had an elevated contribution of the direct path (60–70%). The dilution by exchange in the TCA cycle was much the same as in untreated rats, but dilution by nonglucose carbon was lower.

Table 2. Direct path calculated from [U-$^{13}$C]-, [1-$^{14}$C]-, and [3-$^{3}$H]glucoses

| Rat | Pretreatment | Direct path, % | | | Ratios | |
|---|---|---|---|---|---|---|
| | | $^{13}$CU | $^{14}$C1 | $^{3}$H3 | $^{14}$C6/ $^{14}$C1 | $^{3}$H3/ $^{14}$C1 |
| 1 | None | 52 | 50 | 51 | 0.07 | 0.94 |
| 2 | None | 50 | 51 | 51 | 0.06 | 0.84 |
| 3 | Glucagon | 69 | 70 | 73 | 0.06 | 0.96 |
| 4 | Glucagon | 60 | 66 | 68 | 0.08 | 0.89 |

Data are from experiments of Table 1. Abbreviations are as in Table 1.

**Gluconeogenesis.** In previous experiments (1) and those of Table 1, the calculated enrichment in blood glucose ranged from 94% to 101%, averaging 98%, of that in the infusate. However, gluconeogenesis was not abolished by the glucose load. The fraction of labeled molecules of mass $M_1$, $M_2$, and $M_3$ ranged from 12% to 18% of that of all labeled molecules, $M_1$ to $M_6$. However, the contribution of the molecules of smaller mass to enrichment was only from 5% to 8%. The fraction of glucose arising by recycling may be calculated by Eq. 7 (see the *Appendix*), $(0.5\,\Sigma_1^3 M)/\Sigma_1^6 M$. The factor of 0.5 in the numerator arises because, in the conversion of $P$ molecules of triose phosphate containing $Q$ labeled molecules to hexose phosphates, $P/2$ molecules are formed containing, however, $Q$ labeled molecules. [In our previous study (2), we erroneously neglected the factor of 0.5 and overestimated recycling.]

On the average, 8% of blood glucose was recycled. Hence, since the infusion of glucose was 30 mg per min per kg, 0.08 × 30 or ≈2.4 mg of glucose was formed by gluconeogenesis. The mean rate of glycogen synthesis of control rats was ≈0.4 $\mu$mol per min per g of liver or, for a liver content of 3.5% of body weight, about 2.5 mg per min per kg. The rate of gluconeogenesis of a 250-g starved rat is 8–10 mg per min per kg (11). Although our values are rough approximations, it appears that in spite of the large infused glucose load, net gluconeogenic flux into glucose 6-phosphate of about 5 mg per min per kg is 50%–65% that of starved rats. Roughly 50% of the hexose 6-phosphate flux goes into glycogen and about 50% goes into circulating glucose.

## DISCUSSION

**Direct Path.** As illustrated in Fig. 1, this pathway is determined from the relative concentration of the intact glucose in glycogen. This applies to all types of tracers, to glucoses labeled with $^{14}$C, $^{13}$C, $^{2}$H, or $^{3}$H. With labeled carbon, the direct path was correctly measured with [1-$^{13}$C]glucose in the series of studies by Shulman *et al.* (7–9) and with [1-$^{14}$C]- and [U-$^{13}$C]glucose in the present study. With infused [$^{3}$H]glucose, Lang *et al.* (12, 13) have correctly determined the contribution of the direct path by using [3-$^{3}$H]glucose or [6-$^{3}$H]glucose, respectively. The C6/C1 or the $^{3}$H/$^{14}$C activity ratios bear little relation to the magnitude of the pathways. The ratios depend largely on the dilution of the glucose carbon in the indirect path. The values published for the randomization of $^{14}$C in glycogen and the $^{3}$H/$^{14}$C activity ratios vary widely. The cause for this variability has rarely been investigated.

Our concern in this study is the rational analysis of the methods to study the pathways of glycogen synthesis. The

Table 3. Dilution of the three-carbon pool

| Exp. | Pretreatment | Relative enrichment of $^{13}$CU, % | Specific activity of $^{14}$C1, % | Direct path, % | Dilution | | |
|---|---|---|---|---|---|---|---|
| | | | | | TCA (*E*) | Nonglucose (*F*) | Total (*E* × *F*) |
| 1 | None | 65 | 58 | 51 | 1.4 | 2.7 | 3.7 |
| 2 | None | 68 | 60 | 51 | 1.5 | 2.1 | 3.1 |
| 3 | Glucagon | 85 | 86 | 71 | 1.3 | 1.5 | 2.0 |
| 4 | Glucagon | 78 | 76 | 65 | 1.4 | 1.3 | 1.8 |
| 5 | None | 68 | | 52 | 1.4 | 2.2 | 3.3 |
| 6 | Glucagon | 75 | | 60 | 1.5 | 1.8 | 2.7 |
| Mean | None | 59 | — | 49 | 1.4 | 2.7 | 3.8 |
| | | (53–65) | | (37–56) | (1.3–1.5) | (2.1–3.7) | |

Experiments 1–4 are from Table 1. The mean is of nine experiments that include six experiments from ref. 2. *E* and *F* are calculated as shown in the *Appendix*. The range is shown in parentheses.

metabolic regulation of the contributions of the pathways is beyond the scope of this study. Our results show that with animals, when liver glycogen can be sampled, the simplest method to measure the direct path is with hydrogen-labeled glucose. With [3-$^3$H]glucose there may be a loss of tracers by way of the pentose cycle and in recycling between fructose 6-phosphate and fructose 1,6-bisphosphate (14). In our conditions such a loss appears to be very small, if any. However, the use of [5-$^3$H]- or the widely available [6-$^3$H]glucose appears to be the tracer of choice. When instrumentation is available, the tracer of choice is dideuteroglucose.

The metabolic regulation of the pathways of glycogen synthesis was studied by Lang *et al.* (12, 13) and Rossetti *et al.* (15). The contribution of the direct path in rats depended on the dietary and hormonal status of the rats and the glucose load. In fasted humans the contribution of the direct path was about 50%, but it increased to 70% after ingestion of a meal (16). The fact that, in spite of a large glucose load, or a high carbohydrate diet (15), 50% or more of glycogen is formed from three-carbon compounds constitutes the "glucose paradox."

**Indirect Path.** It is apparent from the C6/C1 ratio in glycogen containing [1-$^{14}$C]- and [1-$^{13}$C]glucose as observed by Shulman (7–9) and us, from the high $^3$H/$^{13}$C ratios, and from the small difference between the enrichment of [U-$^{13}$C]glucose in glycogen and the contribution of the direct path that only a small fraction of the carbon in the indirectly formed glycogen contains label from glucose. The use of mass isotopomer analysis provides an estimate of the sources of dilution. The dilution consists of two fundamentally different types, by exchange of labeled with unlabeled carbon and by the contribution of unlabeled nonglucose carbon.

The dilution, by exchange in the TCA cycle, designated *E*, is of great interest. The parameter $1/E$ is the relative specific activity of PEP from uniformly labeled pyruvate. This relative specific activity and the dilution factor *E* are of major importance to calculate gluconeogenesis from lactate. The value of *E* depends on several parameters of the TCA cycle; the rates of pyruvate carboxylation (designated commonly by *y*), the rate of pyruvate dehydrogenase (designated by *x*), the equilibration of oxaloacetate with fumarate, the recycling by pyruvate kinase, and probably other parameters. Equations relating the relative specific activity of PEP to the parameters of *y* and *x* (17) and to the equilibration between oxaloacetate and fumarate have been presented (2). Determination of these parameters with $^{14}$C-labeled precursors requires elaborate degradation of glucose and the use of multiple tracers. Mass isotopomer analysis provides simply and readily the relative specific activity without regard to any assumptions. The relative specific activity of PEP has been remarkably uniform, 0.7–0.75 that of pyruvate with a correction factor for a dilution of 1.4. It does not appear to be affected by glucagon treatment, which increased the contribution of the direct path. Dilution by nonglucose carbon in our control rats was substantially higher than that by exchange, about 2.4.

The dilution of labeled pyruvate in the TCA cycle is an exchange reaction and irrespective of the dilution of tracer, the net contribution to PEP and hexose phosphates is solely from pyruvate. The mechanism for dilution by way of the TCA cycle is generally understood, and there have been numerous studies to quantitate that dilution, to measure the rate of gluconeogenesis from lactate. On the other hand, the role of nonglucose carbon as a precursor has received little attention. However, Shulman *et al.* (7) in starved rats and Streja *et al.* (18) in humans after a prolonged fast have estimated that as much as half of the gluconeogenic flux must be derived from nonglucose carbon. Streja *et al.* (18) designated this flux as a "chemical" dilution to distinguish it from that by exchange. We provide here a quantitative method to measure this contribution to glycogen synthesis and gluconeogenesis. The source of this carbon may be extrahepatic by glycolysis of muscle glycogen and intrahepatic from gluconeogenic amino acids (alanine, glutamine, serine, etc.) and from glycerol. The extent of this contribution in the presence of a glucose load was unexpected. In glucagon-treated rats, this contribution was reduced, tentatively suggesting that this may be under hormonal control; however, our experiments are yet rather limited.

Our study demonstrates the power of mass isotopomer analysis with [U-$^{13}$C]glucose for the study of glycogen synthesis. It is apparent that a similar approach would serve for the study of gluconeogenesis from lactate and other precursors.

Mass isotopomer analysis yields much more information, which is obtained with much less labor, as compared with the use of $^{14}$C-labeled compounds. The GC/MS method is much more sensitive than the application of $^{13}$C NMR. With GC/MS a sample of 50 μg of material is adequate, permitting studies with serial samples of blood. A major drawback of the studies with $^{13}$C, both NMR and GC/MS, is the high cost of $^{13}$C-labeled compounds. Mass isotopomer studies as described here with rats require about a gram of uniformly $^{13}$C-labeled substrate at the current cost from several hundred to more than one thousand dollars per trial. Hopefully, future development of instrumentation with increased sensitivity and reduction in the price of $^{13}$C will permit the wide use of this technique especially with humans.

## APPENDIX

Relative enrichment in glycogen:

$$\frac{\sum_{i=1}^{6} m_i n_i}{\sum_{i=1}^{6} M_i n_i}. \qquad [2]$$

Direct path:

$$D = m_6/M_6. \quad [3]$$

Dilution of three-carbon pool by exchange in TCA cycle:

$$E = \frac{3\sum_{i=1}^{3} m_i}{\sum_{i=1}^{3} M_i n_i}. \quad [4]$$

Dilution of three-carbon pool by nonglucose carbon:

$$F = \frac{2m_6(1-D)}{\sum_{i=1}^{3} m_i D}. \quad [5]$$

Total dilution:

$$E \times F. \quad [6]$$

Recycling of glucose:

$$\frac{\sum_{i=1}^{3} M_i}{2\sum_{i=1}^{6} M_i}. \quad [7]$$

**Derivation of Eq. 4.** In the conversion of $P$ molecules of [U-$^{13}$C]pyruvate to PEP, $P$ molecules of PEP are formed consisting of a mixture of masses with one, two, and three heavy carbons and a small amount, less than 10%, containing no $^{13}$C. Hence, the total enrichment in pyruvate is $3 \times \Sigma_1^3 m$ and the enrichment in PEP is $\Sigma_1^3 mn$. The equation is an approximation since it neglects the formation of unlabeled PEP. The dilution by nonglucose carbon $F$ is overestimated by this amount so the approximation cancels out in the estimation of total dilution, $E \times F$.

**Derivation of Eq. 5.** The contribution of the direct path may be expressed as

$$D = \frac{m_6}{\left(0.5 \times F \times \sum_{i=1}^{3} m_i\right) + m_6}. \quad [8]$$

The factor of 0.5 arises since the yield of hexose 6-phosphate is only one-half of triose phosphate; $F$ is the dilution of the triose phosphate pool by nonglucose carbon. Eq. **5** is obtained by solving for $F$ from Eq. **8** in terms of $D$, $m_6$, and $\Sigma_1^3 m_i$. Since $F$ is not independently known, Eq. **8** is not useful for the determination of the direct path.

We have previously used in addition to Eq. **3** an expression to calculate the direct path according to Kalderon *et al.* (19):

$$D = \frac{m_6}{\sum_{i=1}^{6} m_i}. \quad [9]$$

This expression is erroneous (compare with Eq. **8**). However, since the value of $F$ in our experiments was about 2.5, due to cancellation of terms, there was little numerical error in our use of the wrong expression. The factor of 0.5 is also required in Eq. **7**. Our omission of the factor of 0.5 was pointed out by Des Rosiers *et al.* (20).

**Note Added in Proof.** A theoretical paper by Des Rosiers *et al.* (20) has come to our attention. They have, using our data in ref. 2, revised our estimates of the direct path, claiming it to contribute 65%–71%, rather than 50% reported in ref. 2. They calculate the contribution of the direct path from a modified form of Eq. **9**:

$$\text{Direct path} = \frac{m_6}{0.5\sum_{i=1}^{6} m_i}.$$

This expression is erroneous in neglecting the dilution of the three-carbon pool (see Eq. **8**) and leads to a large overestimate of the direct path. Their claims of 65–71% direct path (as compared to the estimate in ref. 1 of 50%) exceed the relative enrichments of glycogen and blood glucose (58%) and are thus not tenable.

Des Rosiers *et al.* (20) apply corrections for the recombination of three-carbon compounds to form labeled hexose and corrections for recycling in blood glucose to the calculation of the pathways of glycogen synthesis. Recombination decreases proportionally to the square of dilution and is negligible in our studies. Our calculation of the direct path is not affected by recycling (see text).

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