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G I Shulman, … , R G Shulman, R A DeFronzo

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Research Article

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Mechanism of Liver Glycogen Repletion In Vivo by Nuclear Magnetic Resonance Spectroscopy

Gerald I. Shulman, Douglas L. Rothman, Douglas Smith, Carolyn M. Johnson, James B. Blair, Robert G. Shulman, and Ralph A. DeFronzo

Department of Molecular Biophysics and Biochemistry, and Department of Medicine, Yale University, New Haven, Connecticut 06511; Department of Biochemistry, West Virginia University Medical Center, Morgantown, West Virginia 26506; and Diabetes Unit, Massachusetts General Hospital, Boston, Massachusetts 02114

Abstract

In order to quantitate the pathways by which liver glycogen is repleted, we administered [1-^{13}C]glucose by gavage into awake 24-h fasted rats and examined the labeling pattern of ^{13}C in hepatic glycogen. Two doses of [1-^{13}C]glucose, 1 and 6 mg/g body wt, were given to examine whether differences in the plasma glucose concentration altered the metabolic pathways via which liver glycogen was replenished. After 1 and 3 h (high-dose group) and after 1 and 2 h (low-dose group), the animals were anesthetized and the liver was quickly freeze-clamped. Liver glycogen was extracted and the purified glycogen hydrolyzed to glucose with amyloglucosidase. The distribution of the ^{13}C-label was subsequently determined by ^{13}C-nuclear magnetic resonance spectroscopy. The percent ^{13}C enrichment of the glucosyl units in glycogen was: 15.1±0.8%(C-1), 1.5±0.1%(C-2), 1.2±0.1%(C-3), 1.1±0.1%(C-4), 1.6±0.1%(C-5), and 2.2±0.1%(C-6) for the high-dose study (n = 4, at 3 h); 16.5±0.5%(C-1), 2.0±0.1%(C-2), 1.3±0.1%(C-3), 1.1±0.1%(C-4), 2.2±0.1%(C-5), and 2.4±0.1%(C-6) in the low-dose study (n = 4, at 2 h). The average ^{13}C-enrichment of C-1 glucose in the portal vein was found to be 43±1 and 40±2% in the high- and low-dose groups, respectively. Therefore, the amount of glycogen that was synthesized from the direct pathway (i.e., glucose → glucose-6-phosphate → glucose-1-phosphate → UDP-glucose → glycogen) was calculated to be 31 and 36% in the high- and low-dose groups, respectively. The ^{13}C-enrichments of portal vein lactate and alanine were 14 and 14%, respectively, in the high-dose group and 11 and 8%, respectively, in the low-dose group. From these enrichments, the minimum contribution of these gluconeogenic precursors to glycogen repletion can be calculated to be 7 and 20% in the high- and low-dose groups, respectively. The maximum contribution of glucose recycling at the triose isomerase step to glycogen synthesis (i.e., glucose → triose-phosphates → glycogen) was estimated to be 3 and 1% in the high- and low-dose groups, respectively.

In conclusion, our results demonstrate that (*a*) only one-third of liver glycogen repletion occurs via the direct conversion of glucose to glycogen, and that (*b*) only a very small amount of glycogen synthesis can be accounted for by the conversion of glucose to triose phosphates and back to glycogen; this suggests that futile cycling between fructose-6-phosphate and fructose-1,6-diphosphate under these conditions is minimal. Our results also show that (*c*) alanine and lactate account for a minimum of between 7 and 20% of the glycogen synthesized, and that (*d*) the three pathways through which the labeled flux is measured account for a total of only 50% of the total glycogen synthesized. These results suggest that either there is a sizeable amount of glycogen synthesis via pathway(s) that were not examined in the present experiment or that there is a much greater dilution of labeled alanine/lactate in the oxaloacetate pool than previously appreciated, or some combination of these two explanations.

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Introduction

Numerous studies have established that liver glycogen stores are repleted after a carbohydrate meal. However, the widely accepted mechanism of glycogen repletion by direct incorporation of glucose into glycogen (glucose → glucose-6-phosphate → glucose-1-phosphate → UDP-glucose → glycogen) has recently been questioned by studies (1–4) which suggest that the majority (at least half) of liver glycogen is formed by an indirect pathway possibly involving the sequence: glucose → lactate → oxaloacetate → phosphoenolpyruvate → UDP-glucose → glycogen.

In the present study we have attempted to quantitate the contribution of the direct and indirect pathways to liver glycogen repletion in vivo by administering [1-^{13}C]glucose by gavage into awake 24-h fasted rats and examining the ^{13}C-labeling pattern in hepatic glycogen by ^{13}C-nuclear magnetic resonance (NMR).[1] NMR spectroscopy has the unique capability to determine the ^{13}C-enrichment at each position of the glucose molecule simultaneously in the same sample. Each of the ^{13}C-labeled precursors of glycogen will produce a unique labeling pattern in the glucosyl unit of glycogen. If the hepatic glycogen ^{13}C-labeling pattern and the portal vein ^{13}C-enrichments of glucose, lactate, and alanine are known, the relative contributions of each of these precursors to glycogen repletion can be calculated. Furthermore, since the studies are performed in awake unstressed animals, an integrated picture of whole body glucose homeostasis can be obtained.

Methods

Animals. Male Sprague-Dawley rats weighing between 250 and 300 g were used. They were maintained on standard Ralston-Purina Co. (St. Louis, MO) rat chow and were housed in an environmentally controlled room with a 12-h light/dark cycle. 1 wk before study they were fitted with an internal jugular catheter extending to the right atrium. The catheter was filled with a heparin/polyvinylpolypyrrolidone solution, sealed, and tunneled subcutaneously around the side of the neck to the back of the head. The catheter was externalized through a skin incision and anchored to the skull with a dental cement cap. All rats were fasted for

1. *Abbreviations used in this paper:* NMR, nuclear magnetic resonance spectroscopy; OAA, oxaloacetate.

24 h before study to deplete liver glycogen. At 8 a.m. on the day of study, eight rats were given an intragastric bolus of 33% glucose containing 6 mg/g body wt, under light ether anesthesia which lasted <2 min. The glucose was enriched by 50% with [1-^{13}C]glucose. Four rats were sacrificed at 1 h after glucose ingestion and four after 3 h. A second group of eight rats received a lower dose of glucose, 1 mg/g body wt, that was 99% enriched with [1-^{13}C]glucose. Four rats were sacrificed at 1 h post glucose and four after 2 h. In all studies tracer amounts of [1-^{14}C]glucose were added to the ingested glucose to monitor constancy of the plasma glucose specific activity. Following glucose ingestion, the animals were allowed to roam freely in their cages, while blood samples for plasma glucose and [^{14}C]glucose specific activity were taken at 15–30-min time intervals. At the time of sacrifice, animals were injected with 2% sodium thiamylal (Biotal, Bio-Centic, St. Joseph, MO) (1 ml/kg body weight). The abdomen was quickly opened, and a portal vein blood sample was taken. The liver was then freeze-clamped with aluminum tongs precooled to −77°C with liquid nitrogen and weighed. The time from the onset of anesthesia until freeze clamping of the liver was <90 s. Leg muscle samples were also freeze-clamped using the same method. All tissue samples were frozen at −30°C for subsequent analysis. The amount of the administered glucose load that was absorbed was determined by removing the entire gastrointestinal tract from the stomach to the end of the small intestine, mincing finely, and rinsing with a known volume of 0.9% saline. Following centrifugation, the glucose concentration in the supernatant was determined. Urine samples from the bladder were also obtained at the end of the study and found to contain negligible amounts of glucose.

A group of nine control animals was treated in an identical fashion (i.e., diet, housing, catheter insertion, sacrifice, etc.). In this group no glucose was administered and following a 24-h overnight fast liver and muscle samples were obtained for determination of glycogen concentration.

Analytical procedures. Glucose concentrations in plasma and tissue extracts were determined using a Beckman glucose analyzer (Beckman Instruments, Inc., Fullerton, CA). [^{14}C]glucose in plasma was quantitated as previously described (5). The glycogen was extracted from the liver samples utilizing the KOH extraction, ethanol precipitation method of Somoygi (6). The extracted glycogen was dialyzed extensively against deionized Millipore-filtered water (Millipore Corp., Bedford, MA) containing 0.02% sodium azide and then degraded completely to glucose with Rhizopus amyloglucosidase (20 units/ml) in potassium hydrogen phthalate buffer (50 mM, pH 4.5, at 25°C for 4–6 h). Samples were lyophilized and brought up in 0.5 ml of 99.8% D_2O. The cold glycogen concentration in liver and muscle samples was determined as previously described (7).

NMR methodology. ^{13}C-NMR spectra of glucose were taken at 90.55 MHz in a Bruker WH 360 wide-bore spectrometer or at 125.76 MHz in a Bruker WM 500 spectrometer (Bruker Instruments, Inc., Billerica, MA). Multiple samples, run on both spectrometers, yielded identical results. The samples were placed in standard 5-mm NMR tubes and the spectra were obtained using standard 5 or 10 mm ^{13}C-NMR probes. In both systems a 45° pulse was repeated every 3 s for 1–8 h (3,600–10,000 scans). Spectra were acquired with 4,096 data points over a sweep width of 10,000 Hz centered at 80 ppm (relative to β-D-glucose C-1 at 96.8 ppm) (0.205 ms acquisition time). Broad band proton noise decoupling (2–5 W) centered 1 ppm upfield from water was on only during the acquisition time to avoid the development of the nuclear Overhauser effect. The spectral parameters used allowed the intensities of glucose resonance to be compared without T_1 (relaxation time) and nuclear Overhauser effect corrections. Resonance intensities were determined by computer integration and by manually cutting out and weighing plotted resonances; both methods yielded similar results.

The ^{13}C-fractional enrichment of the C-1 position of each glucose sample was determined from ^{1}H-NMR spectra at 360.13 MHz in a standard ^{1}H-NMR probe. A 45° pulse angle (3 μs) with a 10-s relaxation delay was used. During the relaxation delay, the water and deuterium (HDO) resonance was saturated with a single radio frequency field of 50 mW (8). Spectra were acquired with a sweep width of 5,000 Hz centered at the HDO resonance in 8192 data points (0.819 ms acquisition time). Samples were heated to 340°K to shift the HDO resonance upfield from the C-1 proton resonance of α-D-glucose.

The ^{13}C-fractional enrichment of plasma C-1 glucose, C-3 alanine, and C-3 lactate were determined from ^{1}H-NMR spectra using a homonuclear double resonance spin echo difference sequence (9, 10). A 10-s relaxation delay was used for the glucose and a 15-s delay for lactate and alanine determinations. During the relaxation delay, the HDO resonance was saturated with a 50 mW single radio frequency field (8). During the spin echo τ delay (136 ms for glucose, 68 ms for lactate and alanine), single frequency ^{1}H-decoupling was applied at 50 mW. All other spectral parameters were as described above. The spectral parameters used allowed the intensities of the proton resonances from protons bonded to ^{13}C- and ^{12}C-nuclei to be compared without T_1 or T_2 corrections.

Materials. [1-^{13}C]glucose (99% enriched) was purchased from Cambridge Isotope Laboratories (Cambridge, MA) and [^{14}C]glucose (1 mCi/10 ml) from New England Nuclear (Boston, MA). All other reagents were of the highest quality that was commercially available.

Calculations. A representative ^{13}C-NMR spectrum of the extracted, hydrolyzed hepatic glycogen from a rat which received the high dose (6 mg/g body wt) glucose study is shown in Fig. 1. Eight distinct peaks can be identified and the carbon atoms of glucose have been labeled according to their alpha or beta position. The integrated area under each peak was calculated by an Aspect 2000 computer using DISNMRP 84 software (Bruker Instruments).

The percent labeling (E) for each of the six carbon peaks was computed as follows:

$$E1 = [\beta\text{-C-1} + \alpha\text{-C-1}] \times F$$

$$E2 = [(\beta\text{-C-2})(Z) + \beta\text{-C-2}] \times F = [\alpha\text{-C-2} + \beta\text{-C-2}] \times F$$

$$E3 = [(\alpha\text{-C-3})/(Z) + \alpha\text{-C-3}] \times F = [\beta\text{-C-3} + \alpha\text{-C-3}] \times F$$

$$E4 = [\alpha\text{-C-4}, \beta\text{-C-4}] \times F = 1.1\%$$

$$E5 = [(\beta\text{-C-3}, \beta\text{-C-5}) + \beta\text{-C-2} + \alpha\text{-C-3} + (\alpha\text{-C-2}, \alpha\text{-C-5}) - (\alpha\text{-C-4}, \beta\text{-C-4})] \times \frac{F}{2}$$

$$E6 = [\alpha\text{-C-6}, \beta\text{-C-6}] \times F$$

α-C and β-C are the resonance intensities of the α- and β-C position of glucose, Z represents the ratio of the α-anomer to the β-anomer, and F represents the factor used to normalize the C-4 labeling to 1.1% = E4 (i.e., natural abundance).

All rats were fasted for 24 h to reduce the amount of liver glycogen present at the start of the study. However, a small amount of glycogen still remained and a correction for the unenriched glycogen initially present was applied as follows:

$$\text{Corrected } ^{13}\text{C-labeling} = \frac{(E) \times [\text{glycogen}]_e - (1.1) \times [\text{glycogen}]_i}{[\text{glycogen}]_e - [\text{glycogen}]_i} \quad (1)$$

where $[\text{glycogen}]_e$ is the glycogen concentration at the end of the study and $[\text{glycogen}]_i$ is the glycogen concentration at the start of the study. The above calculation was performed for C-1, C-2, C-5, and C-6 of glucose (see Table I).

To calculate the amount of glycogen synthesized from the direct pathway, the specific [1-^{13}C]glucose labeling of portal venous glucose must be determined. This was accomplished by proton NMR spectroscopy as previously described by Rothman et al. (9). This separated the two smaller ^{13}C-satellite peaks from the larger ^{12}C-peak (Fig. 2). To avoid any overlap with neighboring peaks, the downfield ^{13}C-satellite peak area was used to calculate the percent ^{13}C-labeling in position 1 as follows:

$$\text{Percent } ^{13}\text{C-labeling} = \frac{(2)(\text{C-13})}{(2)(\text{C-13}) + (\text{C-12})} \times 100 \quad (2)$$

The percent glycogen synthesized by the direct pathway can be determined by the following equation:

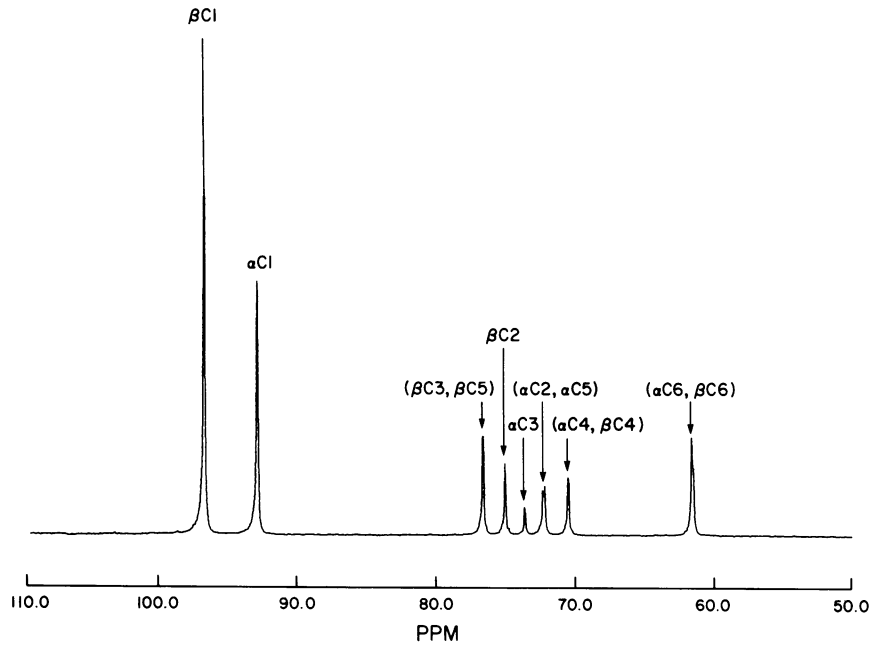

Figure 1. Representative ^{13}C-NMR spectrum of glucose obtained from the hydrolyzed liver glycogen extract. As can be seen, there are eight distinct peaks representing the α and β anomers of C-1–C-6 glucose. Most of the label appears in the C-1 position.

Percent glycogen synthesized via direct pathway

$$= \frac{(E1 - E6)}{(PV\ E1 - 1.1)} \times 100 \quad (3)$$

where E1 and E6 represent the ^{13}C-percent labeling in positions one and six in glycogen at the end of the study, PV E1 represents the percent labeling of C-1 glucose in the portal vein, and 1.1 represents the correction for natural abundance of ^{13}C in portal venous glucose.

To calculate the amount of glycogen synthesized from the alanine/lactate indirect pathway, one must know the specific ^{13}C-labeling in the methyl carbons (position 3) of portal venous lactate and alanine. This was done using homonuclear decoupling techniques (9, 10). This information was used to calculate a minimum value for glycogen synthesis from lactate/alanine:

Percent glycogen synthesized from alanine and lactate

$$= \frac{[(E2 - 1.1) + (E5 - 1.1)]}{(PV\ C\text{-}3 - 1.1)} \times 100 \quad (4)$$

where E2 and E5 represent the ^{13}C-percent labeling in positions two and five of glucose in glycogen at the end of the study, 1.1 represents the correction for natural abundance ^{13}C in C-2 and C-5 of the administered glucose, and PV C-3 represents the average portal vein ^{13}C-percent labeling in position 3 of lactate. The percent labeling of ^{13}C in the three position of lactate (determined on individual samples) was essentially identical to the ^{13}C-labeling in position 3 of alanine (obtained from pooled samples) in all cases.

An estimate of the maximum amount of glycogen synthesized from the triose-phosphate pathway can also be derived:

Percent glycogen synthesized from triose-phosphates

$$= \frac{2(E6 - E5)}{(PV\ E1 - 1.1)} \times 100 \quad (5)$$

Data in the text, table, and figures are given as mean values±SEM. Statistical comparisons were made using the Student *t* test or analysis of variance where applicable.

Results

Figs. 3 and 4 show the time courses for central venous plasma glucose concentration (*top*) and [^{14}C]glucose specific activity (*bottom*) during the high- and low-dose groups, respectively. As can be seen, there was an abrupt rise in plasma glucose concen-

*Table I. Percentage ^{13}C-Labeling of Glucose in Liver Glucose**

	C-1	C-2	C-3	C-4	C-5	C-6	% ^{13}C in C-1 by ^{1}H NMR
High-dose (6 mg/g)							
1 h (n = 4)	11.1 (13.1)±1.8	1.6 (1.7)±0.2	1.2±0.1	1.1±0.1	1.3 (1.3)±0.3	2.0 (2.2)±0.2	11.4±0.2
3 h (n = 4)	14.1 (15.1)±0.8	1.5 (1.5)±0.1	1.2±0.1	1.1±0.1	1.6 (1.6)±0.1	2.1 (2.2)±0.1	15.0±1.5
Low-dose (1 mg/g)							
1 h (n = 4)	13.4 (21.3)±1.7	1.6 (1.9)±0.1	1.3±0.1	1.1±0.1	1.6 (1.9)±0.1	1.9 (2.4)±0.2	12.6±1.0
2 h (n = 4)	12.6 (16.5)±0.5	1.8 (2.0)±0.1	1.3±0.1	1.1±0.1	1.9 (2.2)±0.1	2.1 (2.4)±0.1	12.9±1.5

* All values represent the mean±SEM. Values in parentheses represent corrected ^{13}C-enrichments as described in Eq. 1 where $[glycogen]_i$ = 0.16 g/100 ml, $[glycogen]_{1h}$ = 0.92 g/100 ml, and $[glycogen]_{3h}$ = 2.30 g/100 ml in the high-dose study (6 mg/g); and where $[glycogen]_{1h}$ = 0.41 g/100 ml, and $[glycogen]_{2h}$ = 0.63 g/100 ml in the low-dose study (1 mg/g).

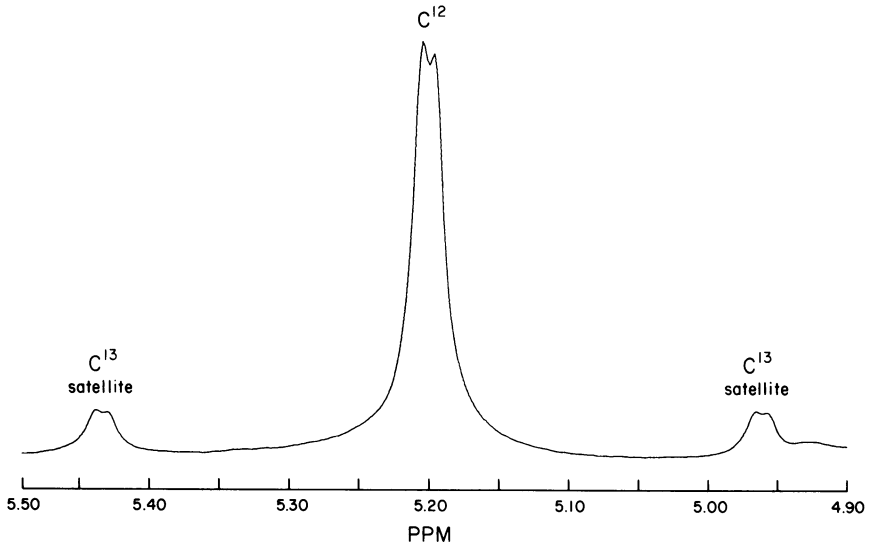

Figure 2. Representative ^{1}H-NMR spectrum of the C-1 position of the glucosyl unit obtained from hydrolyzed hepatic glycogen.

tration and [^{14}C]glucose specific activity within the first 30 min. Thereafter, both remained relatively constant until the end of the experiment.

In Fig. 5 it can be seen that the liver glycogen concentration (0.16±0.03 g/100 ml) in nine control rats after a 24-h overnight fast was quite low. Following the oral glucose load, there was a significant increase in liver glycogen at all time intervals in both the low- and high-dose groups. The rates of glycogen synthesis were linear in both groups and were 0.66 and 0.22 μmol/g liver per min in the high- and low-dose studies, respectively. At the time of sacrifice 82% of the glucose load was absorbed in the high-dose group (at 3 h) while 94% was absorbed in the low-dose group (at 2 h). This was calculated by subtracting the amount of glucose given from the amount that was recovered in the gut and then dividing by the amount of glucose given. The average liver weights at the end of the studies were 9.7±0.3 g (n = 4) and 9.1±0.4 g (n = 4) in the high- and low-dose groups, respectively. The product of the liver weight and the glycogen synthetic rates yields the total amount of glycogen synthesized during the study. Liver glycogen increased by 1,152 and 240 μmol in the high- and low-dose groups, respectively. From these values, it could be calculated that the increase in liver glycogen in the high- and low-dose groups, respectively, accounted for 15 and 18% of the administered glucose load.

Muscle glycogen concentration increased from 0.55±0.05 g/100 ml (n = 9) in the postabsorptive state to 0.67±0.07 g/100 ml (n = 4)(P < 0.05) at 1 h to 0.83±0.07 g/100 ml (n = 4)(P < 0.01) at 3 h in the high-dose group. There was no detectable change in the muscle glycogen concentration in the low-dose group. Assuming that the muscle mass accounts for 40% of body weight in the rat, and the average rat weight was 284±4 g (n = 4), then ~23% of the glucose load could be accounted for by the increase in muscle glycogen in the high-dose group.

Table I shows the average percent labeling of C-1 through

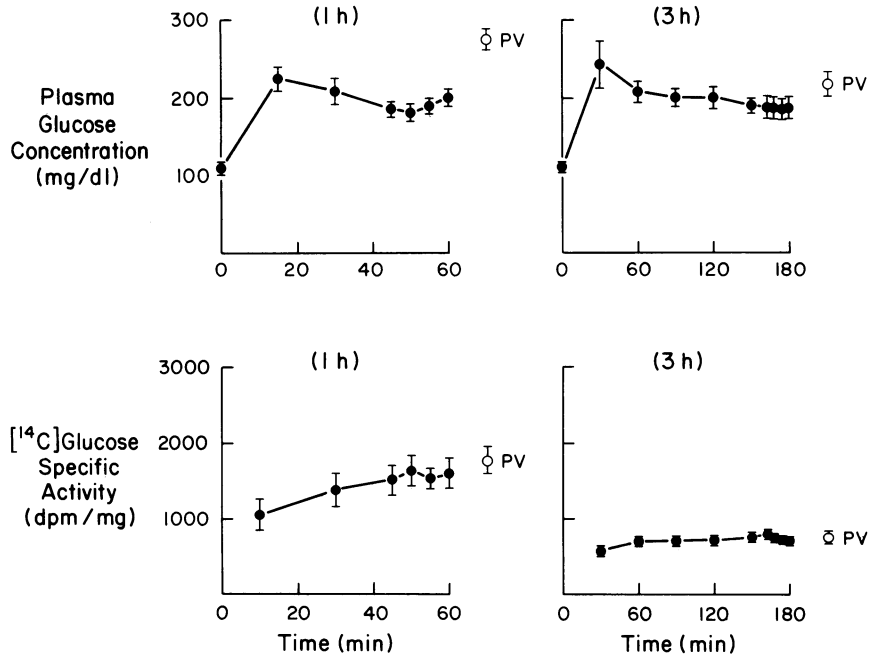

Figure 3. Time course of change in mixed venous plasma glucose concentration (*top*) and mixed venous [^{14}C]glucose specific activity (*bottom*) during the high-dose (6 mg/kg body wt) glucose study. The values represent the mean±SEM of four animals sacrificed at 1 h after oral glucose (*left*) and of four animals sacrificed 3 h after glucose (*right*). Portal venous glucose concentrations and specific activities determined at the time of sacrifice are indicated by the open circles.

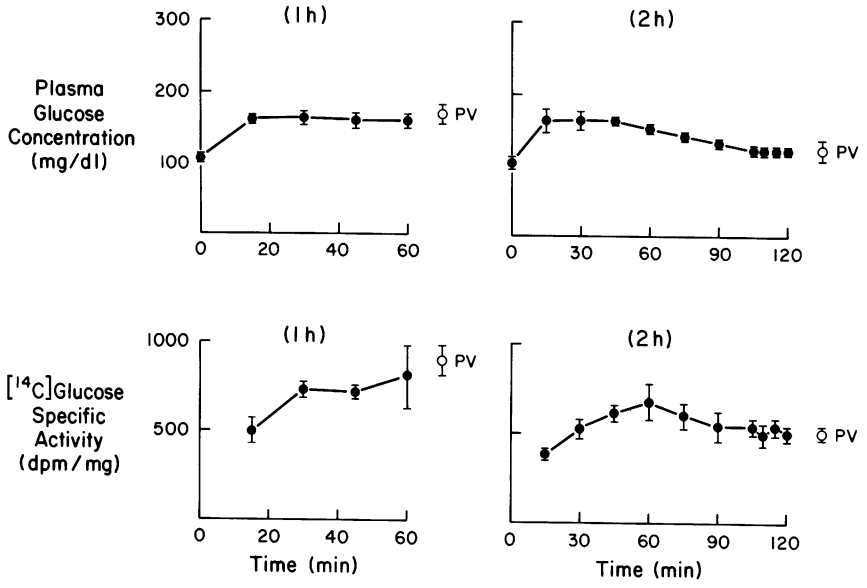

Figure 4. Time course of change in mixed venous plasma glucose concentration (*top*) and mixed venous [^{14}C]glucose specific activity (*bottom*) during the low-dose (1 mg/kg body wt) glucose study. The values represent the mean±SEM of four animals sacrificed at 1 h (*left*) and 2 h after oral glucose (*right*). Portal venous glucose concentrations and specific activities determined at the time of sacrifice are indicated by the open circles.

C-6 in both the high- and low-dose groups. In the calculations, as discussed above, the percent labeling in C-4 was set equal to 1.1%. This assumed that an insignificant amount of label gets incorporated into C-3 or C-4 from the [1-^{13}C]glucose load. To test this assumption, ^{13}C-percent labeling in C-1 position of glucose was independently measured using ^{1}H-NMR. As can be seen in Table I, proton NMR yielded values that were in very close agreement to those obtained by assuming that the C-4 position had 1.1% ^{13}C.

To quantitate the contribution of the direct pathway to the synthesis of glycogen, it is necessary to determine the ^{13}C-percent labeling of glucose in the portal vein. This was done in all animals and found to be 43±1 and 42±2% at 1 and 3 h, respectively, in the high-dose group, and 46±2 and 33±3% at 1 and 2 h, respectively, in the low-dose group (see Table II).

The average [^{13}C]glucose percent labeling over the 3 h of the high-dose study was 43% and over the 2 h of the low-dose study was 40%. Using Eq. 3 it can be calculated that 31 and 36% of the liver glycogen was synthesized by the indirect pathway in the high- and low-dose groups, respectively. Thus, approximately two-thirds of the glycogen was synthesized by indirect pathway(s).

^{13}C-percent labeling of lactate and alanine in portal venous blood was determined by ^{1}H-NMR (Table II). The average ^{13}C-percent labeling for lactate was quite similar to that for alanine and the percent labeling for both was approximately one-third of that for glucose. Using these values, a minimum contribution of alanine and lactate to glycogen formation can be calculated from Eq. 4. In the high- and low-dose studies, a minimum of 7±2 and 20±1%, respectively, of newly synthesized glycogen

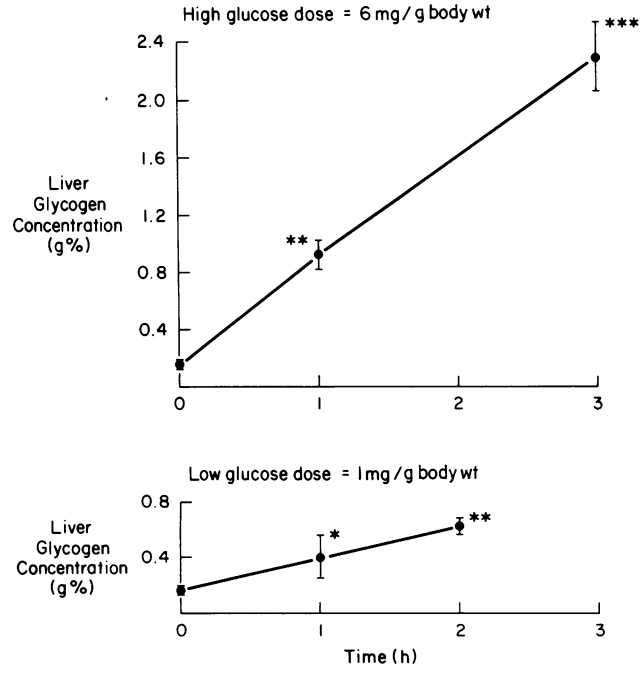

Figure 5. Time course of increase in liver glycogen concentration following the glucose load in the high (*top*)- and low (*bottom*)-dose studies. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared with glycogen concentration at $t = 0$.)

*Table II. Percentage ^{13}C-Labeling of C-1 Glucose, C-3 Lactate, and C-3 Alanine in the Portal Vein**

	1 h (n = 4)	3 h (n = 4)
	%	%
High-dose (6 mg/g) (n = 8)		
Percent ^{13}C C-1 glucose	43±1	42±2
Percent ^{13}C C-3 lactate	14±1	13±1
Percent ^{13}C C-3 alanine	17	12
	1 h (n = 4)	2 h (n = 4)
Low-dose (1 mg/g) (n = 8)		
Percent ^{13}C C-1 glucose	46±2	33±3
Percent ^{13}C C-3 lactate	12±2	10±1
Percent ^{13}C C-3 alanine	8	8

* All values represent the mean±SEM. The percentage of ^{13}C in the C-3 position of alanine represents pooled samples from each group. The percentage of ^{13}C in the C-3 position of lactate represents the mean of individual values for each rat.

was derived from alanine plus lactate. These represent minimum values because labeled oxaloacetate (OAA) derived from these gluconeogenic precursors is diluted by unlabeled OAA derived from the TCA cycle (11). In fed rats it has been determined that this dilution factor is 1.38 (12). Using this value the contribution of alanine/lactate to glycogen synthesis can be estimated to be 10 and 28% in high- and low-dose groups, respectively.

The maximum contribution of glucose recycling at the triose isomerase level to glycogen synthesis was also calculated from Eq. 5 and found to be very small, representing only 3 and 1% in the high- and low-dose groups, respectively.

Discussion

Following glucose ingestion or intravenous glucose administration in both man and animals, liver glycogen concentration has been shown to increase (13, 14). However, studies employing the hepatic venous catheterization technique have demonstrated that insufficient amounts of glucose are taken up by the splanchnic region to account for the repletion of hepatic glycogen stores (2). The demonstration that fructose is a more efficient precursor than glucose for liver glycogen synthesis (13) provided one of the earliest clues that the gluconeogenic pathway may be the predominant mechanism via which hepatic glycogen is replenished. This simple sugar can be converted to glycogen only after it has entered the cell, been phosphorylated, and cleaved to triosephosphates (14). Several recent publications have further emphasized the importance of gluconeogenic precursors in the formation of liver glycogen (1, 3, 4, 15–17).

In the present study we have employed NMR spectroscopy to provide a quantitative estimate of the pathways via which hepatic glycogen is synthesized. The principle of this technique is based upon the ability to define the labeling pattern of ^{13}C in the various positions of glucose in glycogen following the administration of [^{13}C]glucose labeled in the C-1 position. Thus, if glucose were converted directly to glycogen (glucose → glucose-6-P → glucose-1-P → UDP-glucose → glycogen), all of the ^{13}C-label should appear in the C-1 position. Alternatively, if glucose is first catabolized to triose phosphates at the isomerase level, which are subsequently converted to glycogen, equal amounts of label would be found in the C-1 and C-6 positions. Lastly, if glucose were converted to lactate/alanine/pyruvate and these three carbon compounds served as the precursors for glycogen formation, one would expect equal amounts of ^{13}C-label in positions C-1, C-2, C-5, and C-6. We have referred to the latter two pathways by which glycogen is formed as the indirect or gluconeogenic pathways. Our results indicate that only one-third of liver glycogen is synthesized by the direct route, while the other two-thirds comes via the indirect pathways. These conclusions, however, are based upon the following assumptions: (*a*) there is minimal hepatic pentose cycle activity under the experimental conditions employed; (*b*) there is negligible incorporation of the label into the C-3 and C-4 positions of the glucosyl units of glycogen; (*c*) there is rapid and complete equilibration of label between dihydroxy-acetone phosphate and glyceraldehyde-3-phosphate at the triosephosphate isomerase step; and (*d*) there is rapid and complete equilibration of label between the C-2 and C-3 positions of OAA coming from equilibration between malate and fumarate. Each of these assumptions will be discussed below.

If glucose were to enter the pentose cycle, the ^{13}C-label in C-1 would be cleaved and $^{13}CO_2$ would be formed. This would lead to an underestimation of the amount of glucose that was directly incorporated into glycogen. The activity of the pentose cycle has been examined both in vitro (18) and in vivo (19) and found to represent no more than 10% of the total glucose flux in both circumstances. Furthermore, other workers (4, 20) found that the ratio of the specific activities of [^{14}C]glucose in liver glycogen to administered [^{14}C]glucose were nearly identical whether [1-^{14}C] or [6-^{14}C]glucose were administered. These data support the assumption that the activity of the pentose cycle in the liver is small compared with the total glucogenic flux under these circumstances.

Our analysis also assumed that the incorporation of ^{13}C-label into the C-3 and C-4 positions of glucose in glycogen is negligible. This was, in fact, checked by ^{1}H-NMR analysis of the labeling of the C-1 glucose position. This value agreed closely with the percent enrichment determined by ^{13}C-NMR. This agreement implies that the amount of ^{13}C-label in positions C-3 and C-4 must be close to 1.1% natural abundance enrichment.

With respect to assumption *c*, most studies have shown that there is a rapid and essentially complete equilibration between dihydroxy-acetone phosphate and glyceraldehyde-3-phosphate at the triose isomerase reaction (21, 22). Furthermore, our own data support the assumption in that the enrichment of C-2 was equal to the enrichment of C-5. To the extent that isotopic equilibration is not complete, the amount of label in the C-1 position will be slightly overestimated while that in the C-6 position will be slightly underestimated. The net result will be an underestimation of the contribution of the "triose" pathway and an overestimation of the direct pathway to glycogen synthesis.

Lastly, to the extent that isotopic equilibration between the C-2 and C-3 positions of OAA is not complete, the contribution of the gluconeogenic pathway to liver glycogen repletion will be underestimated. This underestimation would occur because C-2 and C-5 would have less label than if equilibration were complete. However, most studies that have examined this question have found almost complete equilibration of the label between the C-2 and C-3 position of OAA when labeled lactate, alanine, or pyruvate is used as the precursor (18, 23, 24). Even if there is incomplete equilibration, the maximum error would be <5%. Assuming incomplete equilibration, minimum percent glycogen synthesized from alanine/lactate equals $(E5 - 1.1) + (E6 - 1.1)/(PV\ C\text{-}3 - 1.1) \times 100$, or 12 and 24% vs. 7 and 20% (assuming complete equilibration) in the high- and low-dose groups, respectively.

Our observation that the direct pathway accounts for approximately one-third of hepatic glycogen repletion is in agreement with the findings of Newgard et al. (3). They infused doubly labeled [3-^{3}H, U-^{14}C]glucose into 20-h starved rats and found that the specific activity of ^{3}H and ^{14}C in newly synthesized glycogen was 12 and 33%, respectively, of the blood glucose. The relative decrease in tritium compared with carbon specific activity can only be explained if glucose was first metabolized to the triose level, at which point the tritium label would be lost. From these results, they concluded that, at a maximum, only 12–28% of hepatic glycogen synthesis could have been derived directly from glucose. In a subsequent publication (4), these same workers examined the scrambling of the ^{14}C-label between C-1 and the rest of the glucosyl units (C-2–C-5) in hepatic glycogen following the intragastric administration of [1-^{14}C]glucose. They found that 39–59% of the ^{14}C-label was recovered in the C-1 position of glucose. This is less than in the present study where 82–87% of the ^{13}C-label was found in C-1. The reason for this

discrepancy is not clear but may be related to differences in the diet, age of the rats, method of glucose administration (bolus vs. continuous infusion), lighting conditions, or actual length of fast. Newgard et al. (4) suggested that the dose of glucose might play an important role in determining the pathway via which glycogen is replenished following feeding. Their results suggested that higher glucose loads (4 mg/g body weight), with resultant higher portal venous glucose levels, preferentially favored the direct pathway by activating the more sluggish, high capacity, low affinity glucokinase reaction. However, our results showed no significant difference in the amount of glycogen synthesized by the direct pathway in the low (36%)- vs. high (31%)-dose studies. Spence and Koudelka (20) also found no difference in the percentage of glycogen synthesized by the direct pathway when cultured liver cells were incubated with a high vs. low medium glucose concentration.

Several earlier studies have examined the labeling pattern of glucose in glycogen following the administration of [1-^{14}C]-, [1-^{13}C]-, [2-^{14}C]-, or [6-^{14}C]glucose either intragastrically or intravenously (19, 25–29). Our results agree with these data in that most of the label in glycogen was found in the same position as the administered glucose. While Newgard et al. (4) have shown that anesthesia might explain the lack of randomization in some of these studies, it would not explain our results or those of others (25–28), who employed animals that were not anesthetized. Because of this observation, previous investigators have concluded that glycogen repletion occurs primarily via a direct pathway (i.e., without passage through three carbon intermediates). It should be emphasized, however, that without knowledge of the portal venous glucose specific activity, the actual amount of glycogen synthesized via the direct pathway cannot be determined. Our study extends these earlier observations in that the specific activity of glucose in the portal vein was measured and found to be high relative to that of other glycogen precursors (such as alanine and lactate). Therefore, the total amount of glycogen derived from the direct pathway is small, amounting to no more than one-third of total glycogen formation. Our results also point out another problem with prior studies that have attempted to define the pathways via which glycogen is repleted. For alanine/lactate, the specific activity in portal vein is approximately one-third that of the administered glucose and, consequently, the amount of ^{13}C-label recovered in positions C-2–C-6 is quite small and may be difficult to quantitate. Furthermore, if precursors other than alanine/lactate/pyruvate contribute to glycogen repletion, no label will be detected in positions C-2–C-6.

The present study design allowed us to quantitate the minimum contribution of lactate and alanine to glycogen synthesis (these can be considered together since their ^{13}C-enrichments are approximately equal; Table II). This was found to be 7±2% in the high-dose group and increased significantly to 20±1% ($P < 0.01$) in the low-dose group. As pointed out earlier, these numbers represent an underestimate because of dilution of the label as it passes through the OAA pool. Hetenyi (12) has experimentally calculated a dilution factor of 1.38 in fed rats. Using this value, the contribution of lactate and alanine to glycogen synthesis becomes 10 and 28% in the high- and low-dose groups, respectively. However, caution is required in applying this factor to our calculations since the metabolic state of our animals was different from that of Hetenyi's. In addition, Hetenyi's correction factor is based on a model assuming a negligible amount of pyruvate dehydrogenase (PDH) activity. This is likely to be an untenable assumption, especially under the hyperinsulinemic conditions of our study.

Glycogen synthesis from the indirect pathway, involving glucose conversion to triose-phosphates and subsequent conversion to glycogen, was also calculated and found to represent 3 and 1% of hepatic glycogen synthesis in the high- and low-dose groups, respectively. This is a maximum estimate because the transaldolase reaction can exchange label between the triose pool and the bottom half of fructose-6-phosphate. The ratio $2 \times (E6 - E5)/(E1 - E5)$ provides a maximum measure of futile cycling between fructose-6-phosphate and fructose-1,6-diphosphate (30), and indicates that only a small amount of substrate (<10%) cycling occurs at this step under the present experimental conditions. This observed scrambling of label into C6 is in good agreement with the results of Van Schaftingen et al. (31).

If one sums up the total amount of glycogen synthesis from the direct and indirect (both lactate/alanine and triose phosphates) pathways, one can account for only 44 and 65% of the newly synthesized glycogen in the high- and low-dose groups, respectively. Thus, in both groups, about half of glycogen synthesis cannot be accounted for. Two possibilities could explain these results. First, the flux of unlabeled acetyl coenzyme A entering the TCA cycle following glucose ingestion may be much greater than estimated by Hetenyi (12). If the correction factor for dilution in the OAA pool were to increase to 4–8, lactate/alanine could explain the majority of the unaccounted for glycogen formation. In fact, in a recent theoretical analysis, Katz (32) has estimated that the correction factor is greater than the 1.38 estimated by Hetenyi (12), but is unlikely to exceed 2–3. Another possible explanation is that a significant fraction of glycogen repletion is derived from unlabeled glycogen precursors other than lactate/alanine/pyruvate, such as glycerol.

It is of interest to speculate upon the source of lactate/alanine that is used for hepatic glycogen synthesis. In preliminary studies, we (Sillerud, L. O., J. B. Blair, R. Dumire, and R. G. Shulman, unpublished observations) have shown that when [1-^{13}C]glucose is added to the isolated perfused liver preparation, the ^{13}C-label is found almost entirely in the C-1 position of glucose in glycogen. Only a trivial amount of label was found in the C-6 position and no label was detected in the C-2 and C-5 positions. The present findings which demonstrate significant ^{13}C-label in C-2–C-6 indicate that glucose must first be metabolized to lactate/alanine by peripheral (i.e., muscle) and gut tissues or perhaps even liver (20), and that these three carbon compounds then serve as the precursors for glycogen formation. This may have important implications for the diabetic patient in whom peripheral resistance to the action of insulin is a characteristic feature. If insulin-mediated glucose uptake by muscle is impaired, one might expect that this would secondarily lead to a defect in hepatic glycogen synthesis. Thus, the defects in muscle and hepatic glucose metabolism would be complementary and both would contribute to the insulin resistance observed in diabetic subjects.

In summary, our results demonstrate that: (*a*) only one-third of liver glycogen repletion is derived from the direct pathway in which glucose is converted directly to glycogen; and that (*b*) only a very small amount of newly synthesized glycogen would be accounted for by the indirect pathway involving glucose → triose-P → glycogen; this suggests that futile cycling between fructose-6-phosphate and fructose-1,6-diphosphate is quite small but yet detectable under the present experimental conditions. Our results, also, demonstrate that (*c*) alanine and lactate account for

a minimum of between 10 and 28% of the glycogen synthesized; and that (*d*) the three pathways through which the labeled flux was measured accounted for a total of only 40–60% of the total glycogen synthesized. The latter observation suggests that either there is considerable glycogen synthesis from unlabeled precursors that were not measured in the present experiments or that the dilution of labeled lactate/alanine in the OAA pool is much greater than previously appreciated, or some combination of these two explanations.

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References

1. Radziuk, J. 1982. Sources of carbon in hepatic glycogen synthesis during absorption of an oral glucose load in humans. *Fed. Proc.* 41:110–116.

2. Katz, L. D., M. G. Glickman, S. Rapoport, E. Ferrannini, and R. A. DeFronzo. 1983. Splanchnic and peripheral disposal of oral glucose in man. *Diabetes.* 32:675–679.

3. Newgard, C. B., L. J. Hirsch, D. W. Foster, and J. D. McGarry. 1983. Studies on the mechanism by which exogenous glucose is converted into liver glycogen in the rat. *J. Biol. Chem.* 258:8046–8052.

4. Newgard, C. B., S. V. Moore, D. W. Foster, and J. D. McGarry. 1984. Efficient hepatic glycogen synthesis in refeeding requires continued carbon flow through the gluconeogenic pathway. *J. Biol. Chem.* 259: 6958–6963.

5. Cherrington, A. D., W. W. Lacy, and J. L. Chiasson. 1978. Effect of glucagon on glucose production during insulin deficiency in the dog. *J. Clin. Invest.* 62:664–677.

6. Somogyi, M. 1934. The solubility and preparation of phosphorus and nitrogen-free glycogen. *J. Biol. Chem.* 104:245–253.

7. Keppler, D., and K. Decker. 1974. Glycogen: Determination with Amyloglucosidase. *In* Methods of Enzymatic Analysis. H. U. Bergmeyer, editor. Verlag Chemie Weinheim, Academic Press, New York. 1127–1131.

8. Campbell, I. D., C. M. Dobson, G. Jeminet, and R. J. P. William. 1974. Pulsed NMR methods for the observation and assignment of exchangeable hydrogens: application to bacitracin. *FEBS Lett.* 49:115–119.

9. Rothman, D. L., F. Arias-Mendoza, G. I. Shulman, and R. G. Shulman. 1984. A pulse sequence for simplifying ^{1}H NMR spectra of biological tissues. *J. Magn. Reson. Medicine.* 60:430–436.

10. Rothman, D. L., K. L. Behar, H. P. Hetherington, and R. G. Shulman. 1984. Homonuclear ^{1}H double-resonance difference spectroscopy of the rat brain in vivo. *Proc. Natl. Acad. Sci. USA.* 81:6330–6334.

11. Krebs, M. A., R. Hems, M. J. Weidemann, and R. N. Speake. 1966. The fate of isotopic carbon in kidney cortex synthesizing glucose from lactate. *Biochem. J.* 101:242–249.

12. Hetenyi, G., Jr., and C. Ferrarotto. 1983. Correction for metabolic exchange in the calculation of the rate of gluconeogenesis in rats. *Biochem. Med.* 29:372–378.

13. Nilsson, L. H., and E. Hultman. 1974. Liver and muscle glycogen in man after glucose and fructose infusion. *Scand. J. Clin. Lab. Invest.* 33:5–10.

14. Hers, H. G. 1976. The control of glycogen metabolism in the liver. *Annu. Rev. Biochem.* 45:167–189.

15. Hems, O. A., P. D. Whitton, and E. A. Taylor. 1972. Glycogen synthesis in the perfused liver in the starved rat. *Biochem. J.* 129:529–538.

16. Shikama, M., and M. Ui. 1978. Glucose diverts hepatic gluconeogenic product from glucose to glycogen in vivo. *Am. J. Physiol.* 235: E354–E360.

17. Katz, J., and McGarry, J. D. 1984. The glucose paradox. Is glucose a substrate for liver metabolism? *J. Clin. Invest.* 74:1901–1909.

18. Cohen, S. M., P. Glynn, and R. G. Shulman. 1981. ^{13}C NMR study of gluconeogenesis from labeled alanine in hepatocytes from euthyroid and hyperthyroid rats. *Proc. Natl. Acad. Sci. USA.* 78:60–64.

19. Hostler, K. Y., and B. R. Landau. 1967. Estimation of the pentose cycle contribution to glucose metabolism in tissue in vivo. *Biochemistry.* 6:2961–2964.

20. Spence, J. T., and A. P. Koudelka. 1985. Pathway of glycogen synthesis from glucose in hepatocytes maintained in primary culture. *J. Biol. Chem.* 260:1521–1526.

21. Rose, I. A., R. Kellermeyer, R. Stjerholm, and H. G. Wood. 1962. The distribution of ^{14}C in glycogen from deuterated glycerol-^{14}C as a measure of the effectiveness of triose phosphate isomerase in vivo. *J. Biol. Chem.* 237:3325–3331.

22. Cohen, S. M., S. Ogawa, and R. G. Shulman. 1979. ^{13}C NMR studies of gluconeogenesis in rat liver cells: utilization of labeled glycerol by cells from euthyroid and hyperthyroid cells. *Proc. Natl. Acad. Sci. USA.* 76:1603–1607.

23. Rognstad, R., and J. Katz. 1972. Glucogenesis in the kidney cortex. Quantitative estimation of carbon flow. *J. Biol. Chem.* 247:6047–6054.

24. Walter, P., V. Paetkau, and H. A. Lardy. 1966. Paths of carbon in gluconeogenesis and lipogenesis. *J. Biol. Chem.* 241:2523–2532.

25. Hers, H. G. 1955. The conversion of fructose-1-^{14}C and sorbitol-1-^{14}C to liver and muscle glycogen in the rat. *J. Biol. Chem.* 214:373–381.

26. Cook, M., and Y. Lorber. 1952. Conversion of 1-^{14}C mannose and 1-^{14}C glucose to liver and muscle glycogen in the intact rat. *J. Biol. Chem.* 199:1–8.

27. Taylor, W. R., and R. G. Langdon. 1956. Intestinal absorption of glucose in the rat. *Biochem. Biophys. Acta.* 21:384–385.

28. Bishop, J. S., R. Steele, N. Altszuler, A. Dunn, C. Bjerknes, and R. C. De Bodo. 1965. Effects of insulin on liver glycogen synthesis and breakdown in the dog. *Am. J. Physiol.* 208(2):307–316.

29. Reo, N. V., B. A. Siegfried, and J. H. Ackerman. 1984. Direct observation of glycogenesis and glucagon stimulated glycogenolysis in the rat liver in vivo by high-field carbon-13 surface coil NMR. *J. Biol. Chem.* 259:13664–13667.

30. Rognstad, R., and J. Katz. 1976. Effects of hormones and of ethanol on the fructose-6-P-fructose-1,6-P_2 futile cycle during glyconeogenesis in the liver. *Arch. Biochem. Biophys.* 177:337–345.

31. Van Schaftingen, E., L. Hue, and H. G. Hers. 1980. Study of the fructose-6-phosphate/fructose-1,6-biphosphate cycle in the liver in vivo. *Biochem. J.* 192:263–271.

32. Katz, J. 1985. The determination of gluconeogenesis *in vivo* with ^{14}C labelled substrates. *Am. J. Physiol.* 248:R391–R399.