Metabolic pathways leading to liver glycogen repletion in vivo, studied by GC-MS and NMR

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A quantitative analysis of the pathways leading to glycogen repletion in rats was conducted. [U-^{13}C]Glucose was administered intra-intestinally into awake fasted animals. The distribution of glucose isotopomers derived from liver glycogen, liver extracts and plasma was performed by GC-MS and ^{13}C NMR. The potential gluconeogenic precursors for liver glycogen, lactate, alanine, glutamate and glutamine, were also analyzed. The amount of glycogen that is synthesized by the direct pathway was found to be 35%. The ^{13}C enrichment of liver lactate, alanine and glucose is similar, indicating that they are the major precursors for liver glycogen synthesis via the indirect pathway. Our results demonstrate that after 24 h fasting, when glucose is supplied, gluconeogenesis from endogenous sources is not shut off.

^{13}C-NMR GC-MS [U-^{13}C]Glucose (Liver) Glycogen repletion

1. INTRODUCTION

The conventional concept that glucose serves as the major substrate and a direct precursor of liver glycogen (glucose ⟶ glucose 6-P ⟶ glycogen) has recently been questioned [1]. Ultimately, it has been suggested that liver glycogen is formed mainly by an indirect pathway from C_3 compounds [2–6] (glucose ⟶ pyruvate ⟶ glucose 6-P ⟶ glycogen). So far, quantitative analyses of the main routes leading to glycogen repletion are scarce.

In this study a quantitative analysis of the pathways leading to glycogen repletion was performed, using stable isotope techniques. ^{13}C labelling pattern and distribution of glucose isotopomers ($^{13}C_6$; $^{13}C_5$, $^{12}C_1$; $^{13}C_4$, $^{12}C_2$; etc.) derived from liver glycogen, liver extracts and plasma were determined by ^{13}C NMR and gas chromatography mass-spectrometry (GC-MS).

^{13}C-labelled metabolites, lactate, alanine, glutamate and glutamine, considered the potential gluconeogenic precursors for liver glycogen repletion, via the indirect route, were also observed by ^{13}C NMR and quantitatively analyzed by GC-MS. Detailed accounts will be published elsewhere.

2. MATERIALS AND METHODS

2.1. *Materials*

[U-^{13}C]Glucose (98% enriched) was prepared in our laboratory from $^{13}CO_2$ (98% enriched) [7].

2.2. *Animals*

Rats (weighing 180–240 g), surgically fitted with a silastic tube inserted into the intestinal lumen, were placed in restraining cages. A solution of [U-^{13}C]glucose (98% enriched, diluted with unlabelled glucose to 36% enrichment), was infused intra-intestinally for 2 h, into awake, 24 h fasted animals (80 mg/100 g body wt per h). At the end of the infusion the rats were anesthetized, portal venous blood taken, and the livers quickly removed and frozen in liquid N_2. Liver glycogen

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was separated and samples used for various determinations.

2.3. *^{13}C NMR measurements*

^{13}C NMR measurements were performed on a Bruker AM-400 MHz Fourier transform spectrometer operating at 100.614 MHz. Proton-broad-band decoupled ^{13}C NMR spectra were obtained in the following spectrometer conditions: 80° pulses, 23.8 kHz spectral width, 16K Fourier data transformation and 2380 accumulations. To reduce effects from dielectric heating and to maintain the sample temperature at about 20°C, power-gated proton decoupling was used.

2.4. *GC-MS*

GC-MS analyses were performed on a Finnigan 4500 quadrupole GC-MS interfaced to an INCOS data system. The mass spectrometer was operated in the electron impact (EI) mode, or in the chemical ionization (CI) mode with isobutane as reactant gas. Samples were injected through the GC-MS inlet system. Measurements of isotopic abundance were made using computer-selected ion monitoring (SIM).

Glucose samples derived from liver glycogen, liver extracts and plasma were analyzed as the trimethylsilyl derivatives, amino acids as trifluoroacetyl *n*-butyl esters and lactate was derivatized to *n*-propylamide *n*-heptafluorobutyrate. The distribution of the isotopomers and atom% enrichment were determined as previously described [8].

3. RESULTS AND DISCUSSION

3.1. *^{13}C NMR spectroscopy*

^{13}C NMR resolved signals from enriched ^{13}C intracellular metabolites of liver extract are depicted in fig.1. The spectrum consists of numerous

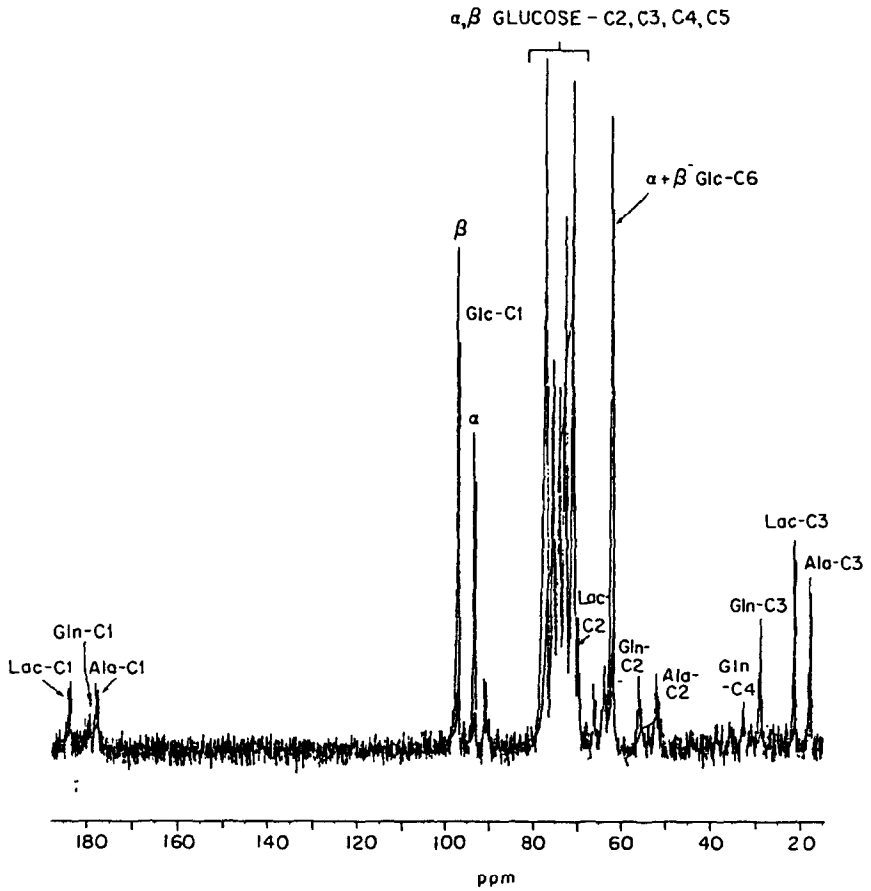

Fig.1. 100.614 MHz ^{13}C NMR spectrum of rat liver extract (free of glycogen).

resonances that have been assigned to glucose, lactate, alanine, glutamate and glutamine, indicating that they are the main glucose metabolites which are the immediate glycogen precursors. The multiplets observed in the ^{13}C NMR spectrum are due to the existence of high percentage of ^{13}C isotopomers having adjacent ^{13}C atoms in the same molecule. Since [U-^{13}C]glucose was diluted with unlabelled glucose, it demonstrates that the glucose metabolites are mainly non-recycled. These results are in accordance with the GC-MS results.

3.2. *GC-MS measurements*

[U-^{13}C]Glucose (98% labelled) was diluted by non-labelled glucose in order to minimize the probability of two labelled three-carbon intermediates joining together to form a glucose molecule. The ^{13}C enrichments of infused glucose (36.1 atom%) and of portal vein plasma, liver extract and liver glycogen were determined by CI GC-MS measurements and are summarized in table 1(a). In various compartments the fraction of isotopomers (P_n) containing n ^{13}C atoms varied as a function of glucose recycling. R is the ratio of glucose molecules having six ^{13}C atoms over the sum of the labelled isotopomer molecules. R values as a function of [U-^{13}C]glucose dilution are depicted in fig.2. A significant decrease of R as a function of increased dilutions of the infused [U-^{13}C]glucose was obtained for glucose derived from different body compartments. R_∞ (obtained at infinite dilution) for glucose of the different origins represents the non-recycled glucose. The R_∞ values obtained were 57% for the portal vein plasma glucose, 20.5% for liver glycogen glucose and 23.5% for liver extract glucose. The non-significant difference of R_∞ values of liver glycogen and liver extract glucoses enabled us to consider the liver glucose isotopomer distribution as reflecting the liver glycogen glucose isotopomer composition. The ratio of R_∞ values of liver glycogen glucose and of portal vein glucose is 35%, indicating that only one third of the plasma [U-^{13}C]glucose contributes to the synthesis of glycogen via the direct pathway. From the ratio of R_∞(portal vein)/R_∞(infused material), it was found

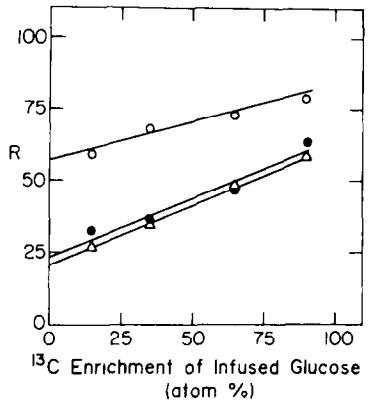

Fig.2. R values (see text) of glucose from different origins as a function of [U-^{13}C]glucose dilutions: glycogen-glucose (△), liver extract (●) and portal vein plasma (○).

Table 1

^{13}C enrichment and isotopomer distribution, calculated from chemical ionization (CI) mass spectra, of glucose in various compartments (a), and ^{13}C enrichment of glycogen and of gluconeogenic precursors in liver extracts (b)

(a)	^{13}C enrichment (atom% excess)	$\sum_{n=1}^{6} P_n$	R
Infused glucose	36.1	36.8	89.4
Portal vein glucose	28.3	34.0	68.5
Liver glucose	21.0	33.9	35.3
Liver glycogen glucose	8.2	13.6	34.5

(b)	Lactate	Alanine	Glutamate	Aspartate	Glucose	Glycogen
Liver	16.5 ± 0.5	16.2 ± 0.8	3.3 ± 0.55	5.0 ± 0.8	21.4 ± 0.9	8.1

that 63% of the plasma glucose is derived directly from the intact molecules of infused [U-^{13}C]glucose, the other 37% of the plasma glucose (and consequently hepatic glycogen) being recycled through glycolysis and gluconeogenesis.

The data summarized in table 1(b) were derived from CI GC-MS measurements. A very important datum is deduced from the ^{13}C enrichments found for lactate or alanine in comparison to hepatic glucose. The calculated ^{13}C enrichment, corresponding to the indirect pathway of liver glucose synthesis, is found to be 16% which is very similar to the enrichment found for lactate or alanine, supporting our ^{13}C NMR results that they are the major source for glucose synthesis via gluconeogenesis. The lower ^{13}C enrichment of glycogen-glucose is a result of pre-steady state glucogen synthesis conditions (further studies are to be published).

In conclusion, the design of the present study enabled us to quantitate the metabolic pathways leading to glycogen repletion after [U-^{13}C]glucose infusion, from the isotopomer distribution of the labelled glucose molecules employing CI GC-MS methodology which avoids separation and degradation methods needed for radioisotope techniques [1–5]. (i) Only 35% of liver glycogen repletion originates from the direct conversion of glucose into glycogen. (ii) The other two-thirds of the glycogen repletion occurs via the indirect pathway, i.e. conversion of glucose to three-carbon units, mainly lactate and alanine and subsequently via glucose 6-P to glycogen. This is the first quantitative demonstration that lactate and/or alanine are the main precursors for the indirect route of glycogen repletion. (iii) Our results indicate that even when glucose is supplied after 24 h fasting, gluconeogenesis from endogenous sources is not shut off. The newly synthesized glucose is mainly directed into liver glycogen, but the liver glucose production is not completely suppressed.

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