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A quantitative analysis of the metabolic pathways of hepatic glucose synthesis in vivo with ^{13}C-labeled substrates

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A quantitative analysis of the major metabolic pathways of hepatic glucose synthesis in fasted rats was conducted. [2-^{13}C]Acetate was administered intraintestinally into awake fasted rats. ^{13}C NMR and GC-MS analysis were used to quantitate the isotopic enrichments of glutamate, glutamine, lactate, alanine and the newly synthesized liver glucose. By measuring the ratio of carbon atoms in glutamate molecules derived from acetyl-CoA to carbon atoms in the glucose molecule derived from oxaloacetate and gluconeogenic substrates, such as lactate and alanine, the relative activities of the Krebs cycle and gluconeogenesis were quantified. Our results indicate that the percentage of glucose carbons originating by 'metabolic exchange' with the oxaloacetate pool, via the Krebs cycle, is less than 7%.

^{13}C-NMR; GC-MS; Acetate; Gluconeogenesis; Krebs cycle; (Liver)

1. INTRODUCTION

Hepatic gluconeogenesis has been the subject of considerable investigations, but the metabolic pathway of gluconeogenesis in vivo, calculated from the transfer of ^{14}C-labeled precursors, remains controversial [1–3]. Recently we have quantitated the main metabolic pathways leading to hepatic glucose synthesis and subsequently glycogen repletion in vivo after [U-^{13}C]glucose administration into awake 24 h fasted rats [4]. Our results demonstrated that lactate and alanine were the major gluconeogenic precursors, as their ^{13}C enrichments were similar to that of the newly synthesized glucose. These results suggest that practically no dilution of lactate and/or alanine occur in the oxaloacetate pool. Under fasting conditions liver glycogen is depleted and the hepatic glucose represents only glucose derived from gluconeogenic origin. By measuring the ratio of carbon atoms derived from acetyl-CoA to carbon atoms derived from gluconeogenic substrates, such as lactate and alanine, in the oxaloacetate molecule, the relative activities of the Krebs cycle and the gluconeogenesis, can be determined (fig.1). Here we present a quantitative analysis of the main pathways leading to glucose synthesis in vivo, following the administration of [2-^{13}C]acetate into fasted rats. A combined method of ^{13}C NMR and gas chromatography-mass spectrometry analysis of liver extracts content, enabled us to quantitate the isotopic distribution and ^{13}C enrichments of glutamate, glutamine, lactate and alanine in comparison to the newly synthesized glucose.

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2. MATERIALS AND METHODS

2.1. *Animals*

Albino rats (weighing 130–150 g) were fasted for 24 h, then a solution of [2-^{13}C]acetate (98% enriched), 1.2 mmol/100 g body wt per h, was infused intraintestinally into 4 awake animals at a rate of 24 μl/min for 2 h as described [4]. At the end of the infusion the animals were anesthetized i.v. with sodium pentobarbital, portal venous

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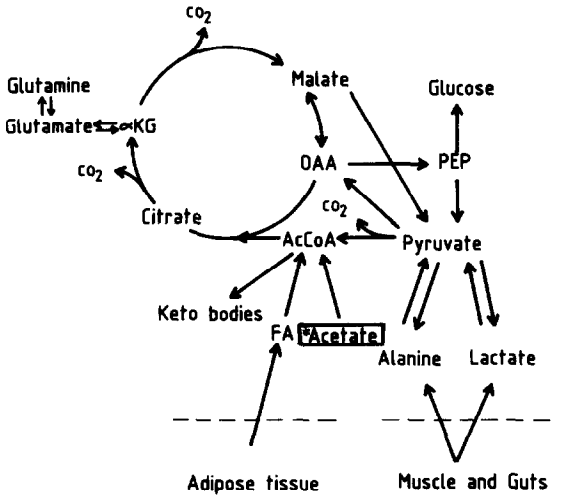


Fig.1. Interrelationships between Krebs cycle and gluconeogenesis in liver from fasted rats. The figure summarizes the possible labelling or dilution pathways, inter- and intracompartmental, of intermediates and end products such as glucose, glutamate, glutamine, lactate, alanine and keto-bodies.

blood samples were taken, and liver and leg muscle were quickly freeze-clamped. Liver extract was prepared as described in [5].

2.2. *Nuclear magnetic resonance measurements*

Proton-decoupled ^{13}C NMR spectra at 67.89 MHz were obtained (at ~10°C) with a Bruker WH-270 MHz spectrometer with the following spectrometer conditions: 60° pulses, 12.5 kHz spectral width, 2 s repetition time, at 16K Fourier data transform and field lock on D_2O. Signal areas were integrated manually and with the aids of the Bruker software. In order to calibrate the intensities of the different ^{13}C peaks of several metabolites, similar ^{13}C NMR spectra of the natural abundance of the corresponding compounds were measured.

2.3. *GC-MS measurements*

GC-MS measurements 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 and in the chemical ionization (CI) mode with isobutane as reactant gas. Measurements of isotopic abundance were made using computer controlled selected ion monitoring (SIM). Glucose was derivatized to 1,2,3,4,6-penta-*O*-trimethylsilyl, α- and β-D-glucose, using Sylon BFT reagent (Supelco Inc.) in pyridine solution (1:1). Two fragments were obtained: m/z 361 (M+1 − 180) corresponds to six glucose carbons and m/z 191 corresponds to the C-1 glucose fragment. Lactate was derivatized to *n*-propylamide *n*-heptafluorobutyrate and the molecular ion m/z 228 (M+1) to 232 (M+4) was monitored by the CI mode. Trifluoroacetyl-*n*-butyl ester of alanine and glutamate were analyzed by the CI mode the molecular ions m/z 242 (M+1) to 245 (M+4) and m/z 356 (M+1) to 361 (M+6) were monitored for alanine and glutamate, respectively. The fragment m/z 152–155 corresponding to (C-2, C-3, C-4) of glutamate was analyzed by the EI mode. The enrichment of C-4 glutamate was calculated from m/z 116–118 [6].

3. RESULTS AND DISCUSSION

3.1. *^{13}C NMR spectroscopy and GC-MS analysis*

Fig.2a shows the ^{13}C NMR spectra of liver extract taken from a fasted rat after [2-^{13}C]acetate administration. The C-2 acetate peak corresponds to some residual nonconsumed acetate. The other intense peaks are those of glutamate and glutamine carbons. C-4 peaks are the most intense, as they are derived directly from [2-^{13}C]acetyl-CoA. Glucose peaks are observed at the region between 60 and 100 ppm. Lactate and alanine ^{13}C resonances are also resolved, and when their NMR spectrum regions were extended their splitting pattern and relative carbon intensities could be analyzed and compared to that of glucose (fig.2b). ^{13}C resonances originating from keto-bodies such as C-2 and C-4 of acetoacetate and C-2 and C-4 of β-hydroxybutyrate, derived directly from [2-^{13}C]-acetyl-CoA, are seen in fig.2a. Fig.2b is the expanded view of the glucose region of fig.2a. Most α- and β-D-glucose anomers are separated and appear as multiplets. The ^{13}C-^{13}C splittings are due to adjacent ^{13}C atoms in the same molecule. The small satellites observed in the ^{13}C spectrum of [^{13}C]glucose are in accordance with the GC-MS study; 15% of [^{13}C]glucose molecules have two carbon-13 atoms in the same molecule. Similar results (15%) were obtained for lactate and alanine. The relative intensities of the different

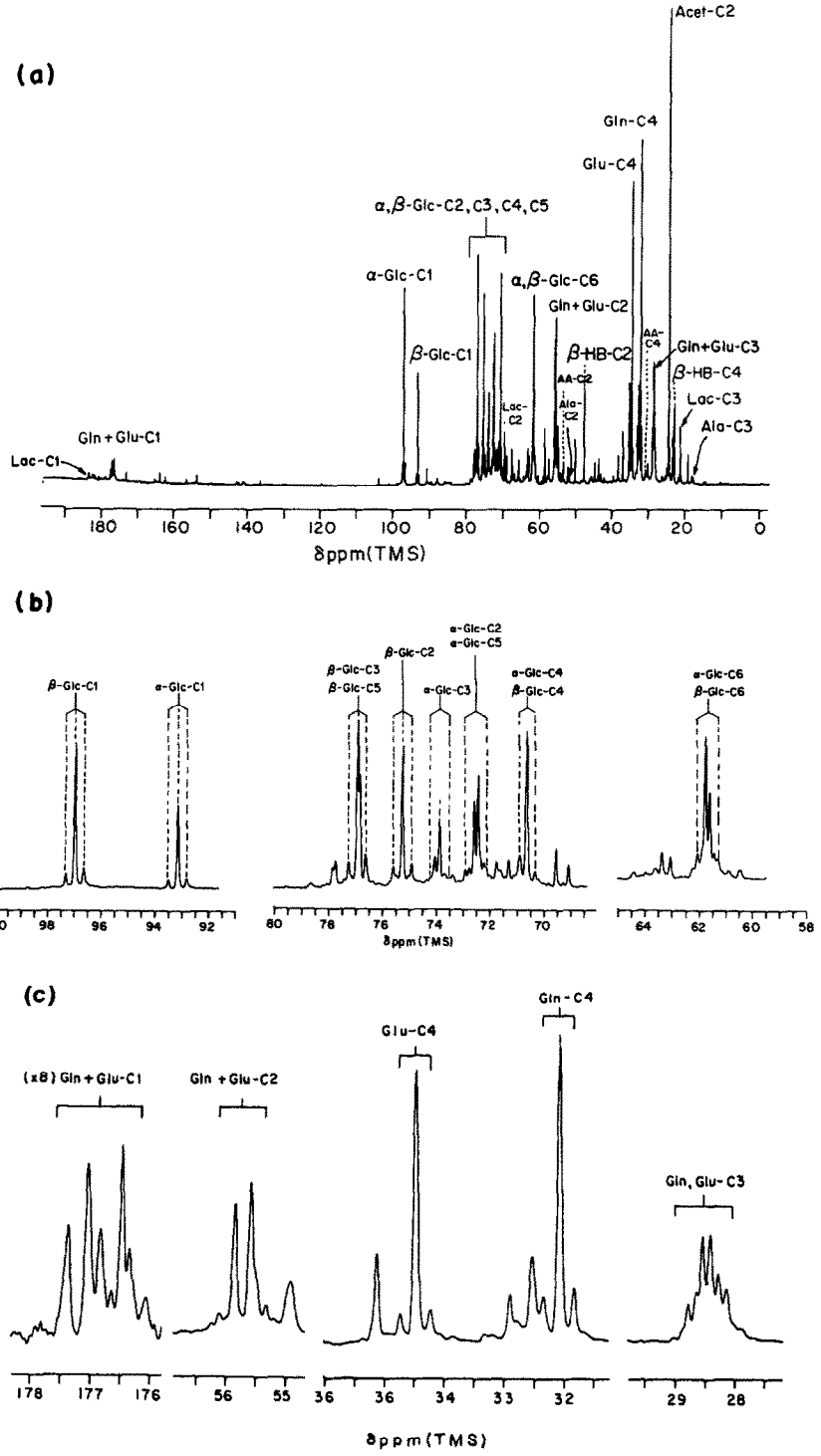


Fig.2. (a) ^{13}C NMR of rat liver extract taken after infusion of [2-^{13}C]acetate. Ala-C3, alanine-C3; Gln + Glu-C3, glutamine-C3 and glutamate-C3; β-HB-C4, β-hydroxybutyrate-C4; AA-C4, acetoacetate-C4; α-Glc-C1, α-glucose-C1, etc. (b) Expanded view of glucose regions of (a). (c) Expanded view of glutamate and glutamine regions of (a).

glucose carbon peaks and a comparison to their calculated ^{13}C enrichments obtained by CI GC-MS allow us to summarize the specific ^{13}C enrichment of each of the six carbon atoms (table 1). The enrichments of C-3 and/or C-4 are 40% less than those of C-1, C-2, C-5 and C-6. A similar variation in enrichment between C-1 and C-3 of lactate and alanine was noted.

Fig.2c is the expanded view of glutamate and glutamine ^{13}C resonances which appear as multiplets due to ^{13}C-^{13}C splitting. The relative ^{13}C enrichments of C-1 through C-4, compared to the specific enrichment of C-4 (18.1%) obtained from mass-spectrometric analysis are summarized in table 1. Resonance corresponding to C-5 glutamate is not observed, as expected in fasting condition, where pyruvate dehydrogenase activity is suppressed [3].

Table 1

The enrichments of [^{13}C]glucose and [^{13}C]glutamate of liver extracts analyzed by GC-MS and ^{13}C NMR spectroscopy

Compound	C atoms	^{13}C enrichments	
		Analyzed by GC-MS	Analyzed by ^{13}C NMR
Glucose[a]	C-1		2.8
	C-2		2.9
	C-3		1.7
	C-4		1.8
	C-5		2.9
	C-6		2.8
Glutamate[b]	C-1	3.1	
	C-2	8.6	8.8
	C-3	8.6	8.8
	C-4	18.1 ± 0.83	18.1 ± 0.83
	C-5	0	0
PEP[c]	C-1	4.3	4.4
	C-2	13.4	13.4
	C-3	13.4	13.4

[a] The ^{13}C enrichment of [1–6-^{13}C]glucose was calculated from GC-MS operating in the CI mode (average carbon enrichment was found to be 2.3 ± 0.3%) and relative ^{13}C NMR intensities

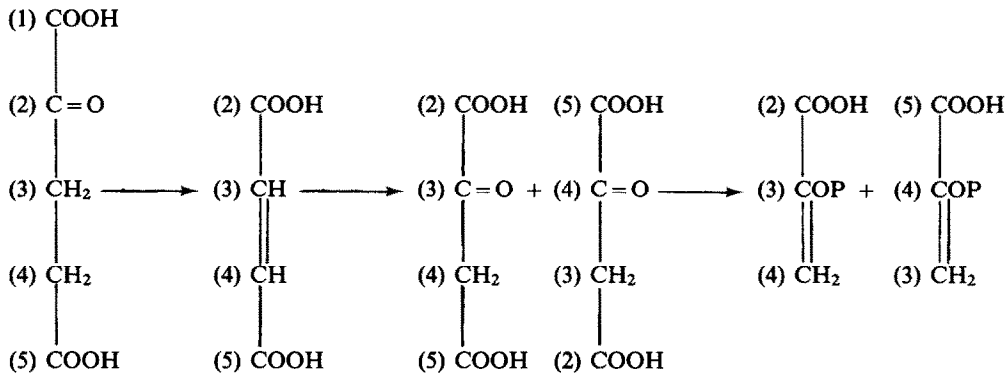
[b] The ^{13}C enrichment of C-4 of glutamate was calculated from GC-MS operating in the EI mode of m/z 116, 117, 118 and was used to calibrate the relative total integrated intensities of the ^{13}C NMR resonances of C-2 and C-3

[c] The ^{13}C enrichment of PEP is derived from carbons 2, 3, 4 and 5 of glutamate. (C-2 + C-5)/2; (C-3 + C-4)/2 and (C-4 + C-3)/2; see scheme 1

3.2. *Quantitative analysis of metabolic pathways*

In the conversion of pyruvate to phospho*enol*-pyruvate (PEP), dilution of oxaloacetate carbons by acetyl-CoA may take place, because oxaloacetate is a common intermediate in the Krebs cycle and in the synthesis of PEP (fig.1). The acetyl carbons of liver acetyl-CoA are assumed not to be available for the net synthesis of liver glucose, but may be incorporated into the glucose by 'metabolic exchange' with the oxaloacetate pool. By measuring the ratio of carbon atoms derived from acetyl-CoA to carbon atoms derived from gluconeogenic substrates in the oxaloacetate molecule, the relative activities of the two pathways can be determined. Oxaloacetate could not be measured because of its low concentration, instead liver glucose can be used as a probe for the carbon-13 distribution in the oxaloacetate molecule. In the present study glycogen is depleted in the fasted rats to 1–2 mg/g wet liver wt, therefore oxaloacetate carbons are the sole glucose source during the course of the study. On the other hand, the ^{13}C enrichment of glutamate and/or glutamine can be used as a probe of the Krebs cycle flux, as it represents the ^{13}C enrichment of the Krebs cycle intermediate α-ketoglutarate. Since muscle glutamine is mainly channeled to the gut and kidney, liver glutamate is not diluted; moreover, under fasting conditions liver glutamine is even released into the circulation [7]. The ^{13}C enrichments of liver glutamate carbons derived from α-ketoglutarate, which are transferred to PEP and subsequently to glucose, are shown in scheme 1. As seen in scheme 1 and table 1, the ^{13}C enrichment of PEP C-3 (which corresponds to glucose C-1, etc.) derived from glutamate (or α-ketoglutarate), is 3-fold higher than C-1, while the observed ^{13}C enrichment of glucose C-1 is only 40% higher than C-3. These findings indicate that glucose synthesis from glutamate turning through the Krebs cycle is limited.

The average ^{13}C enrichment of the glutamate fragment directed towards hepatic glucose syn-



Scheme 1. From left to right: α-ketoglutarate, fumarate, oxaloacetate, PEP (numbers in parentheses correspond to glutamate carbons).

thesis via PEP is calculated from the enrichments of C-2, C-3, C-4 and C-5 as presented in table 1. The average value obtained is 10.4 atom% excess. When this enrichment was compared to the average ^{13}C enrichment of liver glucose, 2.3%, the percentage of glucose carbons originating by metabolic exchange via the Krebs cycle flux was found to be 22% (table 2).

The transfer of ^{14}C atoms from circulating precursors to plasma glucose was estimated from the ratio of the specific activity of plasma glucose and blood acetoacetate during the infusion of [2-^{14}C]acetate into fasted rats by Hetenyi [8]. By this approach 25% of the carbon atoms of plasma glucose have been estimated to arise from acetyl-CoA. Using Hetenyi's method of calculation and comparing the average ^{13}C enrichment (per carbon) of the [C-4, C-5] fragment of glutamate (9%), which represents the acetyl-CoA ^{13}C enrichment, to the average ^{13}C glucose enrichment (2.3%), the resulting value is 25%. But as we have shown (scheme 1 and fig.1), Hetenyi's analysis missed other metabolic pathways of acetyl-CoA. Moreover, our NMR and GC-MS results indicate that a significant ^{13}C enrichment of liver and muscle lactate and alanine occurs during [2-^{13}C]acetate infusion. Pyruvate, which is in equilibrium with alanine and lactate produced in the muscle, can contribute its labeled carbons to the oxaloacetate pool and via PEP to liver glucose. Liver and muscle alanine or lactate enrichments were found to be 2.2%, which is very similar to that of the liver glucose, 2.3%. Muscle glycolysis and/or Krebs cycle activities are suggested as the ^{13}C source of lactate and alanine for the synthesis of the new hepatic glucose. Therefore, when the contribution of 2.2% ^{13}C enriched lactate and/or alanine is included in the calculation (table 2), the percentage of glucose carbons originating by metabolic exchange with the oxaloacetate pool, via the Krebs cycle, is negligible due to the highly enriched gluconeogenic precursor. To correct this underestimation, the calculation should include gluconeogenic amino acids other than alanine and/or glycerol. These unlabeled precursors should dilute the newly synthesized hepatic glucose to some extent, increasing thereby the carbon-13 enrichment originating from the metabolic exchange via the Krebs cycle. The size of the unlabeled pool is unavailable but can be estimated to be 25% [9].

Table 2

Calculation of the hepatic glucose carbons originating from metabolic exchange with the oxaloacetate pools in the Krebs cycle

Average ^{13}C enrichment (atom% excess)			Percentage of glucose carbons originating from metabolic exchange[a]	
A. Glucose	*B*. Alanine/ lactate	*C*. PEP[b]	*X*	*X'*
2.3 ± 0.3	2.2 ± 0.2	10.4	22.3	1.2

[a] $A = C \cdot X + B(1 - X)$; X and X' were derived for $B = 0$ and $B = 2.2\%$, respectively

[b] See table 1 for calculation

When this dilution is used in our calculation (table 2) the percentage of glucose carbon derived from the metabolic exchange with oxaloacetate pool is 7%. Therefore we conclude that the metabolic exchange value is less than 7%. This value is significantly lower than that calculated by Hetenyi (25%), or the value obtained by us, when the contribution of the enriched alanine and/or lactate is not included in the calculation, 22% (table 2).

Another intercompartmental activity is reflected in the ^{13}C enrichment analysis of the liver content: the significant dilution (18.1%) of C-4 of glutamate, which represents the dilution of the infused [2-^{13}C]acetyl-CoA (98%). This dilution is probably due to fatty acids release from adipose tissue, although pyruvate dehydrogenase activity in the liver cannot be eliminated [3].

In conclusion, employing ^{13}C enriched tracer and ^{13}C NMR in conjunction with GC-MS methodologies, the ^{13}C enrichments of most gluconeogenic metabolites were determined. This is the first in vivo study demonstrating that liver lactate and alanine are ^{13}C enriched from [^{13}C]acetate, and their ^{13}C enrichments were included in the calculation of the metabolic exchange of acetyl-CoA with the oxaloacetate pool. The relative fluxes of the main metabolic pathways leading to the newly synthesized liver glucose in fasted animal were quantified.

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REFERENCES

[1] Hetenyi, G. jr, Layberry, R.A., Foster, D.M. and Berman, M. (1980) Am. J. Phys. 239, E39–E44.
[2] Brosman, J.T. (1982) Federation Proc. 41, 91–95.
[3] Katz, J. (1985) Am. J. Physiol. 248, R391–R399.
[4] Kalderon, A., Gopher, A. and Lapidot, A. (1986) FEBS Lett. 204, 29–32.
[5] Cohen, S.M. (1983) J. Biol. Chem. 258, 14294–14308.
[6] Inbar, L. and Lapidot, A. (1986) Eur. J. Biochem., in press.
[7] Lund, P. (1980) FEBS Lett. 117, K89–K92.
[8] Hetenyi, G. jr and Ferrarotto, C. (1983) Biochem. Med. 29, 372–378.
[9] Christesan, H.N. (1982) Phys. Rev. 62, 1198–1233.