Originals

Use of a new gas chromatograph isotope ratio mass spectrometer to trace exogenous ^{13}C labelled glucose at a very low level of enrichment in man

S. Tissot[1], S. Normand[1], R. Guilluy[2], C. Pachiaudi[3], M. Beylot[1], M. Laville[1], R. Cohen[1], R. Mornex[1] and J. P. Riou[1]

[1] INSERM U. 197, Faculté de Médecine Alexis Carrel, [2] L. E. A. C. M. Faculté de Pharmacie Rockefeller, Lyon, and
[3] Centre de Datation et d'Analyses Isotopiques, Université Cl. Bernard, Villeurbanne, France

Summary. The use of ^{13}C labelled glucose in human metabolic studies has been limited by the high cost of the tracer and the problems of measuring low ^{13}C isotopic abundance in plasma glucose. In the present work we describe a new gas chromatograph-isotope ratio mass spectrometer allowing the measurement of a 0.001 atom % increase in ^{13}C abundance over baseline, on a nanomole glucose sample. Studies were performed in rats and in human subjects. The rate of glucose appearance in 24 h fasted rats using D-[1-^{13}C] glucose as tracer and analysed by this new method was found to be 10.4 ± 0.7 mg·kg^{-1}·min^{-1}, a value 21% lower than that found using D-[6,6-[2]H$_2$] glucose as tracer (13.1 ± 1.1 mg·kg^{-1}·min^{-1}) analysed by classic gas chromatography-mass spectrometry. The new method was also used to trace, in combination with D-[6,6 [2]H$_2$] glucose, the metabolic fate in human subjects of two oral glucose loads (0.5 g·kg^{-1}, 1 g·kg^{-1}) la-

belled with 0.1% D-[U-^{13}C] glucose. During the six hours following the glucose load, it was found that total glucose appearance was 0.97 ± 0.04 g·kg^{-1} and 1.2 ± 0.04 g·kg^{-1}, exogenous glucose appearance was 0.51 ± 0.02 g·kg^{-1} and 0.84 ± 0.04 g·kg^{-1}, endogenous glucose production was 0.44 ± 0.04 g·kg^{-1} and 0.35 ± 0.06 g·kg^{-1} after the 0.5 and 1 g·kg^{-1} load respectively. These values are similar to those reported using glucose labelled with radioactive isotopes. These results show that reliable kinetic parameters of glucose metabolism can be determined, without health hazard, in humans, at low cost, using ^{13}C labelled glucose analysed with a new gas chromatograph-isotope ratio mass spectrometer.

Key words: Stable isotope, [^{13}C] glucose, mass spectrometry, human, oral glucose load, gas chromatography.

The metabolic fate of an oral glucose load in humans remains an open scientific question in 1989 [1–7]. This could be due, at least in part, to the absence of convenient, non-hazardous methodology allowing simultaneous measurement of endogenous glucose production and exogenous glucose appearance rate. The increasing availability of glucose labelled with ^{13}C and the development of automated, user-friendly mass spectrometers opens new perspectives in this field.

The isotopic abundance of ^{13}C labelled glucose is measurable either by selected ion monitoring gas chromatography-mass spectrometry [8] or by isotope ratio mass spectrometry (IRMS) after conversion of the glucose molecule to pure CO_2 [9]. The first method is limited by the low sensitivity (0.5–1%) in the measurement of the enrichment [10]. Owing to this limitation, experiments in adults are expensive and bolus injection techniques are debatable because the size of the bolus will disturb the metabolic pool under study. The second method, IRMS, can detect enrichment in ^{13}C in CO_2 as low as 0.001 atom % over natural abundance [11]. This increase can be defined as atom % excess (APE). How-

ever, when the method is applied to an organic compound, a fairly large quantity of the ^{13}C sample is required and it has to be converted into CO_2 in a catalytic furnace before analysis [12]. In order to ensure that the measured enrichment of CO_2 reflects that of the tracee (for example glucose), this compound has to be extensively and completely purified, a method which is usually time-consuming, tedious and sometimes difficult. Therefore, in order to avoid the use of radioactive tracers in the study of glucose metabolism, there is a need [13] for a mass spectrometer which can measure trace amounts of ^{13}C in a very small amount of completely purified glucose.

To provide an answer to this problem, we have combined the purification power of capillary gas chromatography to purify plasma glucose and the sensitivity of IRMS in the detection of low enrichment in ^{13}C, a furnace being interfaced between the two devices. This new design was validated by analysis of prepared chemical samples and by studying, in both rats and man, glucose turnover using D-[6,6-[2]H$_2$] glucose and ^{13}C glucose as tracer.

Subjects, materials and methods

Materials

D-[6,6-^{2}H$_2$] glucose (79.6 mole % excess) was obtained from Tracer Technologies Inc (Somerville, Mass., USA). Chemical and isotopic purity were confirmed by gas chromatography selected ion monitoring mass spectrometry analysis. It was dissolved in sterile isotonic saline (9 g·l^{-1}) and passed through a 0.22 µm Millipore filter (Millipore Corp., Bedford, Mass., USA) before infusion. The preparation was pyrogen free. Its actual concentration was determined at the end of each test.

Pure maize glucose was from Aguettant (Lyon, France) and beet glucose was obtained from Roger Bellon (Neuilly/Seine, France). D-[1-^{13}C] glucose (89.8 mole% excess) and D-[U-^{13}C] glucose (90.5 mole% excess) were obtained from the Commissariat à l'Energie Atomique (Gif-sur-Yvette, France).

In vivo study in rats

Five male Sprague Dawley rats (IFFA Credo, L'Arbresle, France) weighing 216 ± 10 g (mean ± SEM) were studied. Catheters were inserted in the jugular vein and carotide artery ten days before the experiment. They were studied while conscious after 24 h of fasting. D-[6.6-^{2}H$_2$] glucose (0.12 to 0.15 mg·kg^{-1}·min^{-1}) and D-[1-^{13}C] glucose (0.011 to 0.015 mg·kg^{-1}·min^{-1}) were perfused through the jugular vein for 120 min after a priming dose (ten times the infusion rate over 1 min). Arterial blood (0.6 ml) was withdrawn at 90, 105 and 120 min. Plasma glucose concentration and the enrichment in ^{13}C and ^{2}H$_2$ were determined as described below.

In vivo study in human subjects

Subjects. Six normal healthy subjects (age: 26 ± 3 years, body weight: 63 ± 9 kg, body mass index: 22 ± 1.5 kg/(m)^2) were studied. None had a family history of diabetes or had taken any drug during the previous 15 days. They were asked to continue their normal diet but intake of food containing cane sugar, maize starch or exotic fruits was minimized for seven days before the test. The last evening meal contained a fairly large amount of carbohydrate (125 g). Each subject received 0.5 and 1 g·kg^{-1} body weight oral glucose, in a randomized order, with an interval of at least one week between the tests.

Each subject gave written consent to the study after being informed about its nature, purpose and possible risks. The scientific protocol was reviewed and accepted by the INSERM ethical committee.

Protocol

All tests were performed in the postabsorptive state 12 h after the last evening meal, between 07.00 and 08.00 hours, after at least 30 min of bed-rest. Intravenous catheters were inserted into forearm veins for tracer infusion, on one side, and blood sampling, on the other.

A primed-continuous infusion of D-[6,6-^{2}H$_2$] glucose (60 µg· kg^{-1}·min^{-1}) was used for the isotopic determination of glucose appearance and disappearance rates before the glucose load and during the 6 h following the glucose absorption. The priming dose was 80 times the infusion rate over 1 min. Blood samples were taken at 120, 135 and 150 min. Thereafter, 0.5 g or 1 g·kg^{-1} of maize glucose enriched with 0.1% D-[U-^{13}C] glucose was ingested after dilution in 150 ml or 300 ml of water. Blood samples were then taken each 15 min for 90 min each 30 min for the following 90 min and each 60 min thereafter.

Analytical procedures

Metabolites were determined on whole blood collected on ice cold perchloric acid (6% volume/volume (v/v)). Blood glucose, acetoacetate, D-β-hydroxybutyrate and lactate were assayed by standard enzymatic methods [14] as previously described [15]. Ketone body refers to the sum of acetoacetate and D-β-hydroxybutyrate. Plasma immunoreactive insulin and glucagon were determined by RIA [15]. Plasma non-esterified fatty acids were determined by enzymatic methods [16].

In order to measure the isotopic enrichment of plasma glucose, the samples were deproteinized with ice cold perchloric acid (6% v/v) before neuralization with K$_2$CO$_3$ and partially purified by sequential anion-cation exchange chromatography. The neutral eluate fraction was divided in two equal parts. On one part, isotopic enrichment in deuterium was measured. The acetyl-bis-butane-boronyl derivative of glucose was prepared using the method of Bier et al. [17] as previously described [18]. One microliter was injected into a 25 m fused capillary column coated with OV 1701 (Chrompack, Bridgewater, NJ, USA) maintained at 200°C in a gas chromatograph oven (Fractovap 4160, Carlo Erba, Massy, France) and coupled with a mass spectrometer (Quadrupole R 10-10 NERMAG, Rueil-Malmaison, France). The [M-C$_4$H$_9$]$^+$ ion (m/z 297) reflecting unlabelled glucose and the corresponding (M + 2) ion (m/z 299) from the dideutero-glucose tracer were selectively monitored [18]. The peak areas ratio 299/297 was calculated and the corresponding molar ratio was determined from standard curve prepared by mixing weighed amounts of natural and labelled glucose and injected before plasma samples.

On the other half of the neutral eluate, isotopic enrichment in ^{13}C was measured [19]. After lyophilisation, the pentacetate derivative of glucose was prepared by overnight incubation with 50 µl of acetic anhydride (Sigma Chemical, La Verpillière, France) in the presence of 25 µl of pyridine (Pierce, Rockford, Ill., USA). The excess of derivatization reagents was removed by evaporation under nitrogen and the sample was resuspended in 50 µl CHCl$_3$. The enrichment in ^{13}C was measured on the newly developed Isochrom I (European patent applied for n° 87-20-586, 2.09. 1988). Briefly, the sample (1 µl) was injected on a 20 m capillary column coated with OV 1701 maintained at 200°C in a gas chromatograph Oven (Hewlett Packard 9810 A, Evry, France). The gas chromatograph effluent was diverted to a flame ionisation detector until the elution of the pentacetate glucose. The gas chromatograph effluent was then switched inside a catalytic furnace (0.5 mm inside diameter) filled up with CuO and maintained at 800°C. The effluent of the furnace containing CO$_2$ and H$_2$O flowing in the continuous helium flux, was driven in a water trap maintained at – 100°C before reaching the IRMS ion-source (SIRA 12, VG Isogas, Middlewich, UK) where it was ionized. Precise description of the Isochrom I will be given elsewhere. Ions at m/z 44, 45, 46 were continuously recorded until the return of the 44 signal to baseline value. Before and after the CO$_2$ peak arising from glucose pentacetate oxidation, standard CO$_2$ of known enrichment was sequentially injected in the Isochrom I for 30 s 2 to 3 times. The ^{13}C/^{12}C ratio of sample and standard were used to calculate the δ per 1000 ^{13}C value after correction for ^{17}O content (20) using the following equation:

$$\delta\ ^{13}C‰ = [(^{13}C/^{12}C_{sample} - ^{13}C/^{12}C_{standard})/^{13}C/^{12}C_{standard}] \times 10^3$$

The international standard is the Pee Dee Belemnite standard (PDB). The underivatized glucose solutions were also analysed by classic IRMS after complete oxidation at 1000°C in the presence of oxygen and purification of the resulting CO$_2$ by cryogenic distillation [21].

Calculations

The rates of overall (endogenous plus exogenous) glucose appearance (RaT) and disappearance (RdT) were calculated from the enrichment of blood glucose by D-[6,6-^{2}H$_2$] glucose, using the non steady state equation of Steele [22] as modified by De Bodo [23].

Table 1. δ ^{13}C‰ and Atom% ^{13}C of beet and maize glucose (mean ± SEM). Samples for isotope ratio mass spectrometry analysis were prepared by complete oxidation of pure glucose as described in the Methods section. Samples for Isochrom I analysis were derivatized as pentacetate glucose as described

Methodology	Beet glucose	Maize glucose
Isotope ratio mass spectrometry		
δ‰	-25.2 ± 0.15 ($n = 11$)	-12.6 ± 0.11 ($n = 8$)
Atom%	1.08354 ± 0.00017	1.09738 ± 0.00012
Isochrom I		
δ‰	-37.3 ± 0.05 ($n = 10$)	-33.3 ± 0.23 ($n = 6$)
Atom%	1.07019 ± 0.00006	1.07463 ± 0.00025

The determination of the rate of appearance in the systemic circulation of exogenous glucose labelled with ^{13}C has to take into account the fact that in the basal state, plasma glucose is naturally enriched in ^{13}C. Therefore the following variables were determined:

The measured δ ^{13}C ‰ was transformed to ^{13}C atom% (AP) using the following formula:

$$AP = atom\% = \frac{100\,R \times (0.001\,\delta^{13}C_S + 1)}{1 + (R \times 0.001\,\delta^{13}C_S + 1)}$$

where R is the ^{13}C/^{12}C of international PDB standard (R = 0.0112372) and $\delta^{13}C_S$ the δ^{13}C ‰ value of the sample. The AP was always determined on the pentacetate derivative of glucose.

The calculated AP of the ingested glucose and of plasma glucose after ingestion was transformed to APE using the following formula:

$$APE = AP_S - AP_B$$

where AP_S is the AP of the sample and AP_B the AP of the plasma glucose in the basal state (before ingestion). These values were used in the calculation of the rate of exogenous glucose appearance (RaE).

The transposition of the Steele equation suggested by Proietto [24] was used to determine RaE. The formula was transformed for the use of ^{13}C labelled tracer instead of ^{14}C as follows:

$$RaE = \frac{[RaT \times (APE_2 + APE_1)/2] + [pv(G_1 + G_2)/2 \times (APE_2 - APE_1)/(t_2 - t_1)]}{APE\ Glu_{Ing}}$$

where RaT corresponds to the determined total rate of appearance of glucose; APE_1 and APE_2 correspond to the ^{13}C APE at time t_1 and t_2 respectively, G_1 and G_2 are the glycaemia at time t_1 and t_2. APE Glu_{Ing} corresponds to the ^{13}C APE of the ingested glucose. The pool fraction value p was taken as 0.75 and v, the distribution volume was taken as $0.2\,l\cdot kg^{-1}$.

Knowing the RaE, it was possible to determine the rate of disappearance of exogenous glucose (RdE), using the following formula:

$$RdE = RaE - [pv\,(G_1 + G_2)/2 \times (APE_2 - APE_1)/(t_2 - t_1)]/APE\ Glu_{Ing}$$

Knowing RaT and RaE, endogenous glucose production was calculated as:

$$EGP = RaT - RaE$$

Statistical analysis

Results are shown as the mean ± SEM. Statistical significance was evaluated using a Student's t-test for paired data.

Results

In vitro validation of the Isochrom I

In a first set of experiments two different sources of pure glucose (beet glucose and maize glucose) known to have a different enrichment in ^{13}C, maize glucose being more en-

riched [11], were measured by classic IRMS on the underivatized molecule and using the Isochrom I on the pentacetate derivatized molecule. The results are presented in Table 1. As previously reported by others [11], the maize glucose was found, when measured by IRMS, more enriched than the beet glucose. A large difference between the two was also observed when measured by the Isochrom I but the absolute values obtained were much lower. The precision of measurement was in the range of ± 0.1 δ which corresponds to an approximate enrichment of 0.0001 ^{13}C AP.
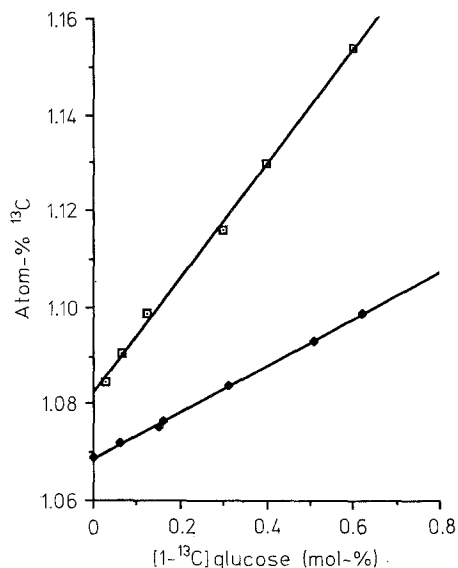

Fig. 1. Standard curves of pure beet glucose enriched with various amounts of D-[1-^{13}C] glucose. The measured enrichment in ^{13}C is expressed as atom %. Each sample was measured by either classic isotope ratio mass spectrometry ($\square$) on the non derivatized glucose molecule or by the new gas chromatograph-isotope ratio mass spectrometer ($\blacklozenge$) on the pentacetate derivative. The equations were respectively: y = 0.118 × + 1.08248, r = 0.999 and y = 0.048 × + 1.06870, r = 0.999

The difference in absolute δ ‰ value or AP observed between both techniques could have been related to either dilution by the exogenous carbon added to the glucose molecule in preparing the pentacetate derivative, or to an isotopic effect related to the increased content in ^{13}C of maize glucose. It can be assumed that the acetic anhydride used in the derivatization procedure has a low ^{13}C content as most chemical products when compared to organic compounds such as glucose [25]. Therefore, the addition of 10 carbons via derivatization, to the glucose molecule should decrease the apparent ^{13}C enrichment of the glucose molecule. On the other hand, if the decreased ^{13}C enrichment observed in glucose measured by the Isochrom I was related to an isotopic effect it can be hypothesized that the expected δ ‰ value for a known enrichment will not be observed and its extent, will not be equivalent to that due to a dilution mechanism by the carbon from the acetic anhydride. In order to identify the correct version of these two possibilities a standard curve of D-[1-^{13}C]glucose diluted in beet glucose was prepared and measured with both techniques. Figure 1 shows that a linear relation was observed between measured ^{13}C enrich-
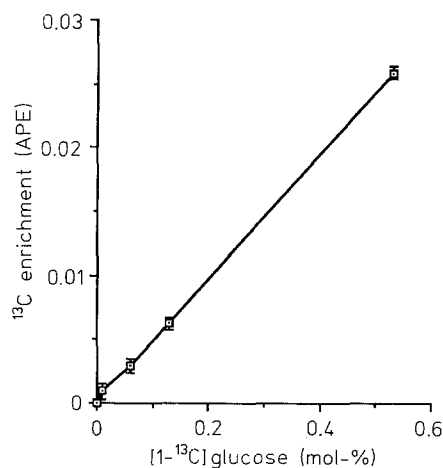
Fig. 2. Standard curve of partially purified plasma glucose enriched with increasing amounts of D-[1-^{13}C] glucose. The measured enrichment in ^{13}C is expressed as APE, i.e. excess of ^{13}C carbon over natural abundance of ^{13}C in derivatized plasma glucose. Each sample was measured three times with the new Isochrom I as a pentacetate derivative. The natural enrichment of derivatized plasma glucose was 1.06945 ± 0.0002, (mean $\pm$ SEM). $y = 0.048 \times + 0.00013$, $r = 0.999$

ment and mol% enrichment even at 0.6 mol% enrichment. Both standard curves join at a theoretical value of 1.06 AP ^{13}C which corresponds to a δ ‰ value of -46.6. This value is commonly found in chemical non-organic products [25]. When the values obtained on the pentacetate derivative of glucose were multiplied by 16/6, the dilution factor introduced by the derivatization process, the values measured by classic IRMS were obtained. Both standard curves differ only by a dilution factor introduced by the derivatization. These data suggest that the lower value observed with Isochrom I measurement is related entirely to the dilution of glucose carbons by exogenous acetic anhydrite carbons added during the derivatization procedure.

The method used was further validated in vitro by preparing a D-[1-^{13}C] glucose standard curve diluted in partially purified plasma glucose (Fig. 2). The samples were not measured by classic IRMS, because the numerous impurities present in the partially purified plasma sample
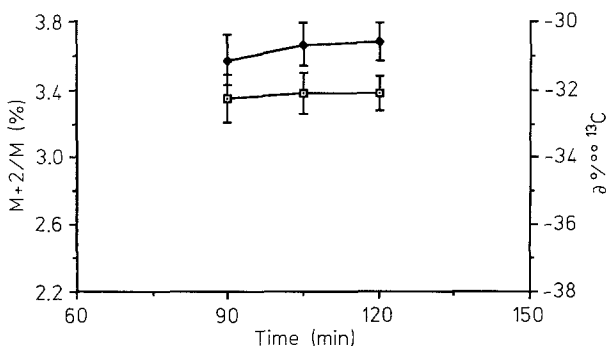
[26] yield erroneous values (data not shown). When this standard curve was measured with the Isochrom I, on the pentacetate glucose derivative, a linear correlation was still observed and the value for the unenriched plasma samples was low. Figure 2 shows that, in a plasma sample, enrichment values as low as 0.001 APE were easily measurable. This corresponds to 0.01 mol% of D-[1-^{13}C] labelled glucose.

In vivo studies

The method used was first validated in rats by measuring glucose turnover rates in the fasted state. Glucose kinetics were measured using both D-[6,6-^{2}H$_2$] glucose and D-[1-^{13}C] glucose as tracer. The enrichment in plasma [6,6-^{2}H$_2$] glucose was determined using classic gas chromatography-mass spectrometry while enrichment of plasma glucose in ^{13}C was determined using Isochrom I. Figure 3 shows that steady enrichment in both tracers was obtained after 2 h of primed constant continuous infusion. The Ra found with D-[6,6-^{2}H$_2$] glucose in five fasted rats was 13.1 ± 1.1 mg·kg^{-1}·min^{-1}. The Ra found with D-[1-^{13}C] glucose was 10.4 ± 0.66 mg·kg^{-1}·min^{-1}. The recycling (Ra [6,6-^{2}H$_2$] glucose – Ra [1-^{13}C] glucose) was 2.27 ± 0.6 mg·kg^{-1}·min^{-1} i.e. $20 \pm 2\%$ of glucose turnover rate (Table 2).

Owing to the sensitivity of this new approach, the method used was tentatively validated by measuring, in humans, the rate of appearance of exogenous maize glucose, enriched with 0.1% D-[U-^{13}C] glucose. The study was performed with both 0.5 and 1 g·kg body weight $^{-1}$ oral glucose load. The total rate of glucose appearance was traced using D-[6,6-^{2}H$_2$] glucose.

Figure 4 shows the evolution of ^{13}C enrichment in plasma glucose during the two different loads in six normal subjects. In both cases, enrichment in ^{13}C of plasma glucose increased steadily for 1 h, almost plateaued during approximately 2 h and slowly decreased afterwards. Six hours after the load ^{13}C in plasma glucose was still significantly higher than baseline values ($p < 0.01$). With the largest load, the increase in ^{13}C in plasma glucose was more
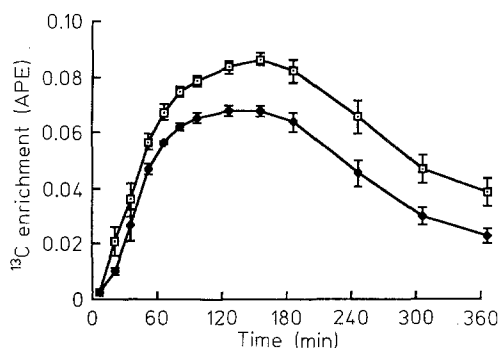

Fig. 3. Isotopic enrichment in plasma glucose of rats obtained after a primed constant infusion of D-[1-^{13}C] glucose (♦) and D-[6,6-^{2}H$_2$] glucose (□) (mean $\pm$ SEM of 5 rats). M + 2/M(%) is the ratio of D-[6,6-^{2}H$_2$] glucose/Natural glucose analysed by GC-MS and δ‰ ^{13}C is the isotopic enrichment in ^{13}C of plasma glucose analysed with the Isochrom I. Measurements of both isotopic enrichments are described in Methods

Fig. 4. Evolution of ^{13}C in plasma glucose during two oral glucose loads (□ 1 g·kg^{-1}; ♦ 0.5 g·kg^{-1}: maize glucose enriched with 0.1% D-[U-^{13}C] glucose) measured with the new Isochrom I as glucose pentacetate. Basal ^{13}C atom % were 1.07042 ± 0.00002 and 1.07023 ± 0.00003 for 0.5 and 1 g·kg^{-1} respectively (mean $\pm$ SEM of six normal subjects)

Table 2. Glucose kinetics in fasting rats

	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5
Glycaemia (mmol/l)	5.77	7.50	7.87	6.47	6.66
Ra (1) (mg·kg^{-1}·min^{-1}) =	10.84	11.92	17.46	12.41	13.13
Ra (2) (mg·kg^{-1}·min^{-1}) =	8.60	10.10	12.51	9.71	10.99
Recycling (mg·kg^{-1}·min^{-1}) =	2.24	1.82	4.95	2.7	2.14
(%) =	21	15	28	22	16

Ra (1) was determined with D-[6,6-^{2}H$_2$] glucose using gas chromatograph-mass spectrometry; Ra (2) was determined with D-[1-^{13}C] glucose using Isochrom I

rapid and the plateau significantly higher ($p < 0.01$). By taking into account the ^{2}H$_2$ enrichment values measured by classic gas chromatography-mass spectrometry and the ^{13}C enrichment values of plasma glucose measured by the Isochrom I, the kinetics of glucose metabolism were calculated (Table 3).

Basal glucose appearance rate averaged 2.20 ± 0.11mg·kg^{-1}·min^{-1}. After ingestion of glucose,

overall glucose appearance increased to a peak value, at 60 min, of 5.6 ± 0.9 and 7.05 ± 0.4 mg·kg^{-1}·min^{-1} for 0.5 and 1 g·kg^{-1} respectively, and returned to basal values between 240 and 300 min. The overall appearance of glucose (RaT) during the 6 h experimental period was 61 ± 3.3 g and 75 ± 4.9 g for 0.5 and 1 g·kg^{-1}, respectively. The overall disappearance of glucose (RdT) increased to a peak value, at 60 min, of 5.1 ± 0.5 and 6.2 ± 1.1 mg·kg^{-1}·min^{-1} for 0.5 and 1 g·kg^{-1} oral glucose load, respectively. In both cases, it returned to the basal value 300 min after ingestion.

Exogenous glucose appeared within 15 min and its appearance rate reached a peak, at 60 min, of 4.1 ± 0.65 and 5.9 ± 0.43 mg·kg^{-1}·min^{-1} for 0.5 and 1 g·kg^{-1} and then, decreased at 0.43 ± 0.05 and 0.65 ± 0.13 mg·kg^{-1}·min^{-1} 6 h after the ingestion of 0.5 and 1 g·kg^{-1}. Exogenous glucose appearance (RaE) accounted for 102% (32 ± 1.1 g or 0.51 ± 0.02 g·kg^{-1}) and 84% (53 ± 2.8 g or 0.84 ± 0.04 g·kg^{-1}) of ingested glucose for 0.5 and 1 g·kg^{-1}, respectively. The rate of disappearance of exogenous glucose (RdE) increased to a peak value, at 60 min, of 2.6 ± 0.6 and 3.7 ± 0.7 mg·kg^{-1}·min^{-1} for 31.5 g and 63 g glucose load. It then decreased to 0.52 ± 0.05 and 0.76 ± 0.14 mg·kg^{-1}·min^{-1} at 360 min.

Table 3. Mean $\pm$ SEM ($n = 6$) of total rate of glucose appearance (RaT), glucose disappearance (RdT), exogenous rate of glucose appearance (RaE) disappearance (RdE), and endogenous glucose production (EGP) in mg·kg^{-1}·min^{-1} during 0.5 g·kg^{-1} (top). 1 g·kg^{-1} (bottom) oral glucose load

Time (min)	mg·kg^{-1}·min^{-1}				
	RaT	RdT	RaE	RdE	EGP
− 30	2.3 ± 0.19	2.3 ± 0.19			2.3 ± 0.19
− 15	2.2 ± 0.11	2.2 ± 0.11			2.2 ± 0.11
0	2.2 ± 0.13	2.2 ± 0.13			2.2 ± 0.13
15	2.9 ± 0.19	2.4 ± 0.20	0.93 ± 0.26	0.12 ± 0.03	2.0 ± 0.21
30	4.1 ± 0.65	2.6 ± 0.29	2.4 ± 0.71	0.75 ± 0.23	1.8 ± 0.23
45	4.8 ± 0.46	2.6 ± 0.50	3.9 ± 0.34	1.7 ± 0.33	0.89 ± 0.18
60	5.6 ± 0.91	5.1 ± 0.55	4.1 ± 0.65[b]	2.6 ± 0.57	1.5 ± 0.28
75	4.3 ± 0.37	4.9 ± 0.54	3.4 ± 0.40	2.6 ± 0.33	0.87 ± 0.13
90	3.4 ± 0.54	4.2 ± 0.16	2.6 ± 0.50[a]	2.2 ± 0.36	0.85 ± 0.09
120	2.9 ± 0.23[a]	4.0 ± 0.36	2.1 ± 0.16[b]	2.1 ± 0.20[a]	0.77 ± 0.10
150	2.2 ± 0.18[a]	2.7 ± 0.40[a]	1.6 ± 0.22[b]	1.8 ± 0.27[a]	0.66 ± 0.07
180	2.2 ± 0.16	2.6 ± 0.19[a]	1.3 ± 0.21[a]	1.4 ± 0.15[a]	0.86 ± 0.11
240	2.1 ± 0.12	2.2 ± 0.16	0.79 ± 0.12[a]	1.1 ± 0.12[a]	1.3 ± 0.14
300	2.0 ± 0.12	2.1 ± 0.22	0.52 ± 0.05[a]	0.80 ± 0.07[b]	1.5 ± 0.11
360	2.1 ± 0.09	2.0 ± 0.11	0.43 ± 0.05	0.52 ± 0.05	1.7 ± 0.11

Time (min)	mg·kg^{-1}·min^{-1}				
	RaT	RdT	RaE	RdE	EGP
− 30	2.2 ± 0.10	2.2 ± 0.11			2.2 ± 0.11
− 15	2.1 ± 0.10	2.1 ± 0.10			2.1 ± 0.10
0	2.0 ± 0.53	2.0 ± 0.10			2.0 ± 0.10
15	3.8 ± 0.81	2.6 ± 0.17	1.9 ± 0.37	0.48 ± 0.15	1.8 ± 0.25
30	4.8 ± 0.40	3.3 ± 0.66	3.1 ± 0.71	1.2 ± 0.31	1.8 ± 0.24
45	5.4 ± 0.71	3.7 ± 0.39	4.7 ± 0.36	2.4 ± 0.09	0.73 ± 0.11
60	7.0 ± 0.36	6.2 ± 1.1	5.9 ± 0.43	3.7 ± 0.72	1.1 ± 0.34
75	4.6 ± 0.29	5.0 ± 0.89	4.0 ± 0.26	2.9 ± 0.34	0.58 ± 0.20
90	4.5 ± 0.43	5.5 ± 0.54	4.2 ± 0.28	3.4 ± 0.43	0.36 ± 0.06
120	4.4 ± 0.55	5.4 ± 0.61	3.9 ± 0.33	3.5 ± 0.54	0.49 ± 0.14
150	4.3 ± 0.55	4.6 ± 0.54	3.8 ± 0.45	3.4 ± 0.45	0.44 ± 0.11
180	2.9 ± 0.25	3.4 ± 0.23	2.3 ± 0.33	2.2 ± 0.19	0.57 ± 0.14
240	2.6 ± 0.17	2.7 ± 0.19	1.6 ± 0.26	1.9 ± 0.24	1.0 ± 0.18
300	2.2 ± 0.08	2.2 ± 0.15	1.01 ± 0.15	1.4 ± 0.15	1.0 ± 0.15
360	1.9 ± 0.10	1.8 ± 0.09	0.65 ± 0.13	0.76 ± 0.14	1.3 ± 0.10

[a] ($p < 0.05$), [b] ($p < 0.01$) indicates statistically significant difference between the two oral glucose loads

Table 4. Mean ± SEM ($n = 6$) of metabolites and hormones after ingestion of an oral glucose load: 0.5 g·kg^{-1} (top), 1 g·kg^{-1} (bottom)

Time min	mmol·l^{-1}				Insulin μU·ml^{-1}	Glucagon pg·ml^{-1}
	Glucose	Lactate	NEFA	KB		
− 30	4.3 ± 0.1	0.45 ± 0.02	0.44 ± 0.03	0.14 ± 0.02	8 ± 1	48 ± 10
− 15	4.2 ± 0.1	0.46 ± 0.03	0.44 ± 0.05	0.15 ± 0.02	7 ± 1	50 ± 9
0	4.3 ± 0.1	0.47 ± 0.02	0.45 ± 0.05	0.16 ± 0.02	8 ± 1	50 ± 10
30	5.4 ± 0.3	0.48 ± 0.02	0.37 ± 0.06	0.18 ± 0.05	27 ± 4	48 ± 9.4
60	6.8 ± 0.3	0.64 ± 0.07	0.13 ± 0.03	0.06 ± 0.01	29 ± 4[a]	38 ± 11
90	6.1 ± 0.3	0.70 ± 0.03[a]	0.06 ± 0.02	0.06 ± 0.01	19 ± 2[b]	41 ± 11
120	5.2 ± 0.3	0.59 ± 0.02	0.04 ± 0.01	0.04 ± 0.01	13 ± 1	38 ± 10
150	4.6 ± 0.2	0.53 ± 0.04[a]	0.13 ± 0.05	0.04 ± 0.01	13 ± 3[a]	43 ± 9
180	4.1 ± 0.2	0.44 ± 0.04[a]	0.26 ± 0.08	0.10 ± 0.02	9 ± 1	47 ± 9
240	3.9 ± 0.1	0.46 ± 0.04	0.51 ± 0.08	0.24 ± 0.09	7 ± 1	49 ± 11
300	3.7 ± 0.2	0.45 ± 0.03	0.67 ± 0.06	0.51 ± 0.11	7 ± 1	53 ± 10
360	3.8 ± 0.2	0.40 ± 0.05	0.61 ± 0.07	0.49 ± 0.11	8 ± 1	54 ± 8

Time min	mmol·l^{-1}				Insulin μU·ml^{-1}	Glucagon pg·ml^{-1}
	Glucose	Lactate	NEFA	KB		
− 30	4.1 ± 0.3	0.50 ± 0.02	0.36 ± 0.09	0.18 ± 0.06	8 ± 1	53 ± 10
− 15	4.2 ± 0.2	0.50 ± 0.03	0.30 ± 0.07	0.19 ± 0.06	8 ± 1	56 ± 10
0	4.3 ± 0.2	0.51 ± 0.03	0.31 ± 0.07	0.18 ± 0.06	9 ± 1	56 ± 11
30	6.0 ± 0.4	0.56 ± 0.05	0.36 ± 0.11	0.18 ± 0.06	38 ± 13	48 ± 8.8
60	7.4 ± 0.5	0.88 ± 0.10	0.08 ± 0.02	0.09 ± 0.02	48 ± 10	42 ± 12
90	6.8 ± 0.6	1.01 ± 0.13	0.03 ± 0.01	0.05 ± 0.01	39 ± 4	42 ± 10
120	5.5 ± 0.6	0.79 ± 0.05	0.06 ± 0.04	0.07 ± 0.02	25 ± 5	44 ± 10
150	4.7 ± 0.4	0.72 ± 0.03	0.04 ± 0.02	0.05 ± 0.01	23 ± 6	39 ± 11
180	4.3 ± 0.4	0.55 ± 0.05	0.14 ± 0.04	0.07 ± 0.01	12 ± 2	46 ± 11
240	4.0 ± 0.3	0.49 ± 0.05	0.28 ± 0.05	0.18 ± 0.08	8 ± 1	54 ± 13
300	4.0 ± 0.1	0.48 ± 0.04	0.58 ± 0.05	0.32 ± 0.13	7 ± 0.5	99 ± 58
360	4.2 ± 0.2	0.46 ± 0.03	0.63 ± 0.07	0.47 ± 0.13	7 ± 0.5	62 ± 19

[a] ($p < 0.05$), [b] ($p < 0.01$) indicates statistically significant difference between the two oral glucose loads (KB refers to ketone body)

Endogenous glucose production decreased, reaching a minimum at 150 min (70% suppression; 0.66 ± 0.07 mg·kg^{-1}·min^{-1}) for 0.5 g/kg and at 90 min (83% suppression; 0.36 ± 0.06 mg·kg^{-1}·min^{-1}) for 1 g·kg^{-1}, and was still suppressed 6 h after investigation: 26% (1.67 ± 0.11 for 0.5 g·kg^{-1} and 39% (1.3 ± 0.1 mg·kg^{-1}·min^{-1} for 1 g·kg^{-1}). Overall endogenous glucose production accounted for 46% (28 ± 2.4 g or 0.44 ± 0.04 g·kg^{-1}) and 29% (22 ± 3 g or 0.35 ± 0.05 g·kg^{-1}) of RaT, during 0.5 and 1 g·kg^{-1} oral glucose load.

The persistance of an apparently significant exogenous glucose appearance rate 6 h after the glucose load, even though all metabolic indices such as glycaemia, lactate and insulin had returned to baseline values as shown on Table 4 was somewhat unexpected. The only difference observed at 6 h compared to baseline was the significantly ($p < 0.01$) higher non-esterified fatly acids and ketone body concentration. The metabolic response was, as expected, different with the two oral glucose loads. The main difference being a significant ($p < 0.05$) higher insulinaemia and lactate during the 1 g·kg^{-1} load. Nevertheless, 4 h after the loads, every metabolic parameter had returned to normal and exogenous glucose appearance was still apparently occurring.

Discussion

The data presented in this work show that the kinetic parameters of glucose metabolism can be traced during an oral glucose load using glucose labelled with stable isotopes exclusively.

The new isotope ratio mass spectrometer developed for this study i.e. a coupling system working on-line between a capillary gas chromatograph, a catalytic furnace and an isotope ratio mass spectrometer, allows the accurate measurement, of trace amounts of ^{13}C (0.0010 ± 0.0002 APE) from a small amount of partially purified plasma glucose (about 10 nmol). Such an approach has been previously reported [27–30] but neither validation in vitro on biological samples nor validation in vivo in human beings or rats have been, to our knowledge, previously reported. The classic IRMS approach for analysis of ^{13}C glucose [12] needs much larger samples (0.1–1 μmol), extensive purification, tedious oxidation of glucose and time consuming CO_2 purification. This new technique solves these problems and speeds up the analysis, one sample being analysed in 10–15 min. It must be emphasized that ^{13}C enrichment in plasma glucose, measured with the Isochrom I, just as when measured with classic IRMS, is in fact a measurement of ^{13}C in CO_2 produced by the complete oxidation of glucose in the catalytic furnace interfaced between the gas chromatograph and the IRMS. Therefore, the measured ^{13}C abundance is a mean of the ^{13}C abundance of each carbon atom from the glucose molecule. Thus, this method does not allow the determination of the site of labelling of glucose as yet.

The derivatization of glucose necessary to analyse its ^{13}C enrichment, also results in a dilution of the glucose carbon with carbon from acetic anhydride. Figure 1 clearly shows that ^{13}C enrichment determined by the Isochrom I is much lower than with classic IRMS. Therefore, the absolute value of ^{13}C in glucose is not measured.

Nevertheless, assuming that ^{13}C enrichment is measured in the same way before, and during ^{13}C labelled tracer appearance, a reliable increase in ^{13}C enrichment can be detected.

The results presented in rats using D-[6,6-$^{2}H_2$] glucose and D-[1-^{13}C] glucose as tracer are similar to published data using ^{3}H and ^{14}C labelled glucose as tracers [31–33]. The lower level of hepatic glucose production using [1-^{13}C] glucose as tracer is consistent with recycling of the carbon skeleton of the glucose molecule. If recycling occurs, it should generate ^{13}C labelled glucose on carbons 1, 2, 5 and 6 [34, 35]. Because the enrichment is measured on the six carbons of the glucose molecule, the measured APE will apparently be increased and thus the rate of appearance decreased.

The validation of the method was also carried out in vivo in humans. The results presented in Table 2 clearly show that the changes in glucose kinetics observed during both oral glucose loads are very similar to those determined using ^{3}H and ^{14}C labelled glucose [5–7]. Therefore, it can be assumed that the derivatization process used in this work does not introduce an artefact in the measurement of kinetic parameters of glucose metabolism.

One intriguing observation of recent work on the metabolic fate of orally administred glucose [5–7] is the persistance, as also observed here (Fig.4), of a significant apparent rate of exogenous glucose appearance at a time at which all metabolic indices are back to normal. This observation has been tentatively related to the existence of late glucose absorption by the gut [5–6, 36] or to the existence of a significant recycling with the release of label from glycogen stores built up early after glucose absorption [5, 7, 37]. It has been shown that most, if not all [4], of the glucose absorption is over, 6 h after the load. Our observation that 100% of the $0.5\,g\cdot kg^{-1}$ load was "recovered" as exogenous glucose argues in favour of this hypothesis.

This unexpected high recovery could be accounted for, at least in part by the recycling of labelled glucose carbon. The extent of activity of this pathway was not measurable in our study as individual enrichments of each glucose carbon could not be determined using the Isochrom I. Nevertheless, our observation that 100% of the $0.5\,g\cdot kg^{-1}$ glucose load had apparently appeared in the peripheral circulation while plasma glucose was still significantly enriched in ^{13}C, supports this hypothesis. Indeed if the peripheral metabolism of ^{13}C labelled glucose produces ^{13}C labelled three-carbon gluconeogenic precursors they will be, if gluconeogenesis is active, transformed first to glucose 6-phosphate and then to labelled glucose which will be artefactually detected as "exogenous glucose" yielding an apparently high recovery of the glucose load [38, 39]. The rate of recycling during an oral glucose load has been estimated to represent 10% of the metabolism of the glucose load [5]. The physiological existence of recycling of plasma glucose during an oral glucose load recently gained large support with the formulation of the theory of the indirect pathway of glycogen synthesis during refeeding [38–40]. In this theory, peripheral glucose metabolism yields three-carbon gluconeogenic precursors which are driven towards glycogen synthesis after being trans-

formed in the gluconeogenic pathway into glucose 6-phosphate. In our experiments it is conceivable that glycogen has been labelled through this pathway. If liver glycogen were degraded during the last hours of the study it would release recycled ^{13}C labelled glucose and therefore contribute to persistent labelling of plasma glucose.

The use of glucose labelled only with stable isotopes in the present study has allowed us to perform sequential experiments with two different oral glucose loads, in the same subjects without health hazard. The comparison of the kinetics of glucose metabolism during this test is of interest. As expected, glycaemic responses were almost identical for the $0.5\,g\cdot kg^{-1}$ (31.5 g) and the $1\,g\cdot kg^{-1}$ (63 g) loads but insulin and lactate responses were significantly increased during the larger load. The differences observed in insulin concentration probably contributed significantly to the observed increased RdT increasing glucose uptake by peripheral tissue and thus explaining the lack of large difference in the glycaemic response. The differences in insulin between the two loads might also affect splanchnic uptake of glucose. Indeed, when this rate was calculated as suggested by Kelley et al. (oral load – RaE) [7], it was found that 10 g of glucose was stored during the 6 h of the experiment during the $1\,g\cdot kg^{-1}$ load and none during the $0.5\,g\cdot kg^{-1}$. These observations are in agreement with most of the recent data on the metabolic fate of glucose [5–7]. Indeed, the observed exogenous Ra $(53 \pm 2.8\,g)$ during the 63 g glucose load compared quite well with the observed exogenous Ra during a 68 g glucose load by Kelley [7] (48 g during 5 h which can be extrapolated to 58 g for 6 h). These results further validate the determination of the metabolism of oral glucose by Isochrom I analysis of ^{13}C appearance in plasma glucose.

In conclusion, our results show that ^{13}C analysis of plasma glucose at ultralow levels of enrichment can easily be measured with a new isotope ratio mass spectrometer allowing a reliable measurement of glucose metabolism in humans at reasonable tracer cost. This new development should have considerable application for the study of intermediary metabolism in man.

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Dr. J. P. Riou
INSERM U197
Faculté de Médecine A. Carrel
8 rue G. Paradin
F-69372 Lyon
France