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Gluconeogenesis in Isolated Rat Hepatocytes Evaluated by Gas Chromatography/Mass Spectrometry Using Deuterated Water

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Tritiated water and radioactive tracers have been used to monitor glucose production by primary cultures of hepatocytes. More recently, ^3H_2O has been replaced for by ^2H_2O in '*in vivo*' studies addressed at the evaluation of the relative contribution of gluconeogenesis to total glucose production. In this work, the possibility of using ^2H_2O to determine the ratio between the glucogenic flux and the overall flux through glucose 6-phosphate in isolated liver cells *in vitro* was evaluated. For this purpose, hepatocytes from either fasted or fed rats were incubated with a medium containing 6, 12 and 25% of ^2H_2O in the presence of either 2 or 20 mM pyruvate. Isotopomer analysis of six different mass clusters (m/z 328, 314, 242, 212, 187 and 145) was carried out by gas chromatography/mass spectrometry (GC/MS) of glucose aldonitrile pentaacetate. For each cluster, ions at $m/z + 1$, $+2$, $+3$ and $+4$ were monitored. From the combination of different clusters the enrichment at C-6 and C-2 of glucose was computed and the C-6/C-2 ratio was considered to represent the contribution of gluconeogenesis to total glucose production, as suggested previously. Based on the results obtained, conditions selected to be optimum for the use of the method in studies on the modulation of gluconeogenesis were as follows: incubation of hepatocytes with 20 mM pyruvate in 12% ^2H_2O followed by GC/electron ionization MS analysis of the clusters of ions at m/z 328, 314 and 187 of the glucose derivative to calculate enrichment at the C-2 and C-6 positions of glucose. © 1998 John Wiley & Sons, Ltd.

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INTRODUCTION

Stable isotopic tracers have been extensively used in studies on turnover rates and/or the metabolism of various compounds.[1] Techniques for estimating the rate of formation of compounds based on incorporation of deuterium from ^2H_2O directly into metabolites have been described.[2,3] In studies on glucose metabolism, methods based on the incorporation of ^2H from ^2H_2O have the advantage over other procedures of incorporation of labeled precursors being independent of the particular precursor used in the enzymatic reaction.

When gluconeogensis takes place in the presence of ^2H_2O, deuterium atoms are incorporated into glucose as shown in Fig. 1.[4] The deuterium distribution among the carbon atoms of newly formed glucose is related to the metabolic step at which incorporation occurs in the glucogenic pathway. As already pointed out by other workers,[5,6] glucose formed from lactate or pyruvate in the presence of ^2H_2O contains deuterium bound to all carbons (Fig. 1). Position 2 of glucose is representative of the whole glucose production because incorporation of deuterium at C-2 occurs both in the synthetic pathways from glucogenic substrates (lactate, pyruvate and trioses) and during glycogenolysis (Fig. 1). The C-6 position is instead considered to be peculiar for gluconeogenesis from lactate and pyruvate being the unique position which is not labeled at the triose level. Therefore, the C-6/C-2 deuterium enrichment ratio represents the gluconeogenic flux relative to the overall flux through glucose-6-phosphate. Underestimation of gluconeogenesis using enrichment at C-6 to calculate glucose formation (10–15%) was attributed[6] to incomplete exchange at position 6 due to the low rate of fumarate–malate interconversion which represents the labeling step for position 6. Therefore, Landau *et al.*[7] recently investigated the possibility of calculating gluconeogenesis using enrichment at C-5. In this case, the formation of glucose from both pyruvate and triose is taken into account. Thus, gluconeogenesis is overestimated owing to the additional labeling at C-5 via the fructose 6P futile cycle.[7,8]

In an *in vivo* investigation, enrichment at C-6 was established by degradation to formaldehyde of glucose obtained from subjects given ^2H_2O, which was then analyzed as the hexamethyltetramine derivative.[6] Enrichment at C-2 was instead determined by the enzymatic transfer of deuterium of position 2 via NADPH to lactate. Guo *et al.*[5] performed instead a single gas chromatographic/mass spectrometric (GC/MS) analysis

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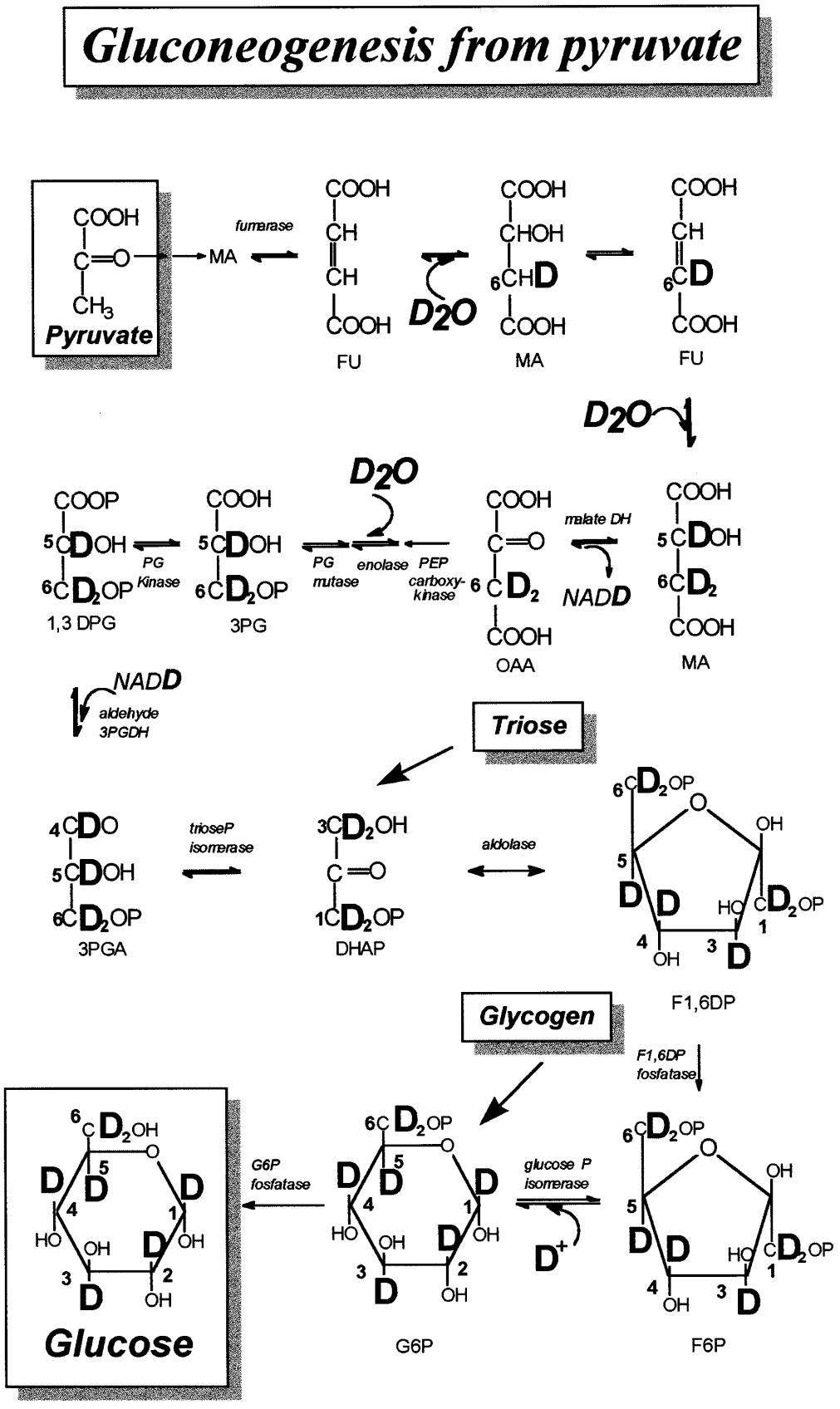

Figure 1. Glucose labeling in the presence of ^2H_2O. Numbers of carbon atoms refer to glucose. Position 6 is the only one irreversibly labeled from pyruvate during fumarate (FU) to malate (MA) equilibration.

of a glucose derivative in which enrichment at C-6 and C-2 was computed on the basis of the enrichment of ions deriving from the fragmentation of the tested derivative of glucose. The latter method allows the complex procedure for the preparation of samples to be avoided but requires higher enrichments than the former method and an accurate choice of the ions to be analyzed. Therefore, use of that method is limited to protocols implying a fairly high enrichment such as those obtained *in vivo* in laboratory animals to which higher amounts of deuterated water can be administered than to humans. To our knowledge, gluconeogenesis in isolated cells has been estimated using radioactive tracers and the glucose degradation technique but the use of ^2H_2O and GC/MS has not been described.

In this paper, we present data on the evaluation of gluconeogenesis in isolated rat hepatocytes by GC/MS measurement of deuterium enrichment of glucose formed during incubation of the cells with ^2H_2O. Optimum enrichment of ^2H_2O to be used in the incubation and isotopomers to be monitored were evaluated under various experimental conditions.

EXPERIMENTAL

Chemicals

[6,6-$^2H_2$]Glucose (99.7% isotopic enrichment) was obtained from Tracer Technologies (Sommerville, USA), unlabeled glucose from NIST (Gaithersburg, MD, USA), ^2H_2O (99.7% isotopic enrichment) from Merck (Darmstadt, Germany), β-D-allose from Fluka (Buchs, Switzerland), Hank's medium from Biospa (Milan, Italy), collagenase and Tripan Blue from Sigma (St Louis, MO, USA) and pyruvate from Boheringer Mannheim (Milan, Italy). All other reagents and solvents were of the highest commercially available purity.

Hepatocyte preparation and incubation

Male Sprague–Dawley rats of 200–250 g body mass (Charles River Italia, Calco, Italy) either fed *ad libitum* or fasted for 24 h (after the last meal) to deplete glycogen[9] were used. Hepatocytes were isolated by a recirculating collagenase perfusion according to Segle's method.[10] In particular, liver was washed with Hank's solution (without Ca^{2+} and Mg^{2+}) for 10 min at a constant flow rate of 15 ml min^{-1}; collagenase (2 mg ml^{-1}) was then added to the medium and liver was perfused for 10–12 min at a constant flow rate of 20 ml min^{-1}. The temperature of the solutions was always kept at 37 °C. At the end of perfusion the liver was placed in cold Hank's solution to block the collagenase activity; cells were washed twice and centrifuged at 500 *g* for 2 min at 4 °C. Cell viability was checked with the Tripan Blue (0.4% solution in water) exclusion test before and after incubation and was 85–95% as described.[9] Hepatocytes (10^6 cells) were suspended in 1.5 ml of RPMI 1640 (without glucose; Seromed, Italy) containing either 2 or 20 mM pyruvate. Water in the medium was enriched at 0, 6, 12 and 25% with ^2H_2O. Cells were then incubated at 37 °C for 60 min under a 95% O_2–5% CO_2 atmosphere. At the end of incubation the samples were centrifuged at 1000 *g* for 2 min and aliquots of the supernatant were collected for glucose analysis.

Sample preparation and GC/MS analysis

Allose (10 or 50 μg) was added to the incubation medium (50–200 μl aliquots) as an internal standard (IS) in order to determine glucose concentration.[11] Calibration curves were prepared by mixing an aqueous solution of standard allose (10 or 50 μg) with various amounts of standard glucose to provide a series of points within an analyte/IS ratio range of 0–2. Samples were deproteinized with CH_3CN and glucose was then converted into aldonitrile pentaacetate as described previously.[12] The mass spectrum of the derivative of allose (not shown) differed from that of glucose [Fig. 2(A)] only in the relative abundances of ions.

GC/MS analysis was carried out in the electron ionization (EI) mode using a Finnigan 4500 mass spectrometer coupled with a Fractovap gas chromatograph (Carlo Erba, Milan, Italy). Separation of glucose from allose was achieved using an SPB-1 fused-silica capillary column (30 m × 0.32 mm i.d., 5 μm film thickness; Supelco, Bellefonte, PA, USA) with helium at 0.2 kg cm^{-2} (head pressure) as carrier gas. Analyses were carried out with increasing oven temperature from 170 °C (held for 1 min) to 270 °C at 35 °C min^{-1} (retention times 5.0 and 5.2 min for allose and glucose, respectively). The injector temperature was kept at 270 °C. The MS conditions were ion energy 70 eV, emission current 0.22 mA, transfer line temperature 280 °C, manifold temperature 70 °C and multiplier voltage 1400–1600 V. The source temperature was set at 140 °C and the pressure was 2×10^{-6}–4×10^{-6} Torr (1 Torr = 133.3 Pa). Under these conditions, glucose analysis was easily accomplished in 10 min. The instrument was tuned daily with perfluorotributylamine.

Selected ion monitoring (SIM) analysis of cluster ions at *m/z* 328, 314, 242, 212, 187 and 145 was carried out with a 50 ms dwell time and a $\pm$0.25 mass unit window. Samples per peak were always >20. The base ion (m0) and ions at $m/z + 1$ (m1), $m/z + 2$ (m2), $m/z + 3$ (m3) and $m/z + 4$ (m4) were monitored for each cluster. The peak area for each ion of the clusters at the retention time of glucose was measured using Incos MSDS rev. 5.5 software. For glucose quantification the ion at *m/z* 187 was monitored and the peak area at the retention times of the derivatives of glucose and allose was calculated.

Calculation

Molar percentage excess (MPE). Measured isotopic ratios (MR_n) were calculated for each ion as peak area ratios: MR_1 (m1/m0), MR_2 (m2/m0), MR_3 (m3/m0) and MR_4 (m4/m0). Similarly, basal isotopic abundances (BR_n) were determined by measuring the isotopic ratios BR_1, BR_2, BR_3 and BR_4 in glucose samples formed by cells incubated in the absence of labeled water. Corrected isotopic ratios for each isotopomer in the cluster (CR_n)

were then calculated as follows:

$$CR_n = MR_n - BR_n - (CR_{n-1} \times BR_1) - (CR_{n-2} \times BR_2) - (CR_{n-3} \times BR_3)$$

where the contribution of basal isotopic abundances of ions n, $n-1$, $n-2$ and $n-3$ (BR_n, $CR_{n-1} \times BR_1$; $Cr_{n-2} \times BR_2$ and $Cr_{n-3} \times BR_3$, respectively) was subtracted from MR_n.

The MPE as a percentage of isotopomer n relative to all isotopomers of each fragment was then calculated[5] as

$$MPE_n = 100CR_n/(\Sigma CR_n + 1)$$

Molar enrichment (ME) and gluconeogenesis. The ME was calculated for each cluster as $\Sigma n \times MPE_n$. Enrichment at C-2 and C-6 of glucose (E2 and E6, respectively) were then calculated as described in the Results section. The gluconeogenic flux relative to the overall flux through glucose 6-phosphate was calculated as $\frac{1}{2}$(E6/E2) $\times$ 100, which represents the percentage of gluconeogenesis as reported previously.[5]

Incubation was carried out in quadruplicate with each cell preparation under each tested condition. MPE, ME, E2, E6 and % gluconeogenesis values were then calculated separately for each sample. The mean, SD and RSD for the cell preparation used were then calculated.

Glucose concentration. The glucose concentration in the medium was calculated from the glucose/allose peak area ratio at m/z 187 (GAR_{187}) as:

$$\text{glucose (mg dl}^{-1}) = (GAR_{187}/b_{187}) \times (1 + \Sigma CR_{n187}) \times DF$$

where b_{187} is the slope of the glucose/allose calibration graph and DF is the dilution factor. In order to take into account all glucose produced (unlabeled and deuterated), the term $(1 + \Sigma CR_{n187})$ was introduced, which considers the complete cluster.

Reproducibility

The imprecision of BR_n, MR_n, CR_n, MPE_n and ME measurements referred to clusters of ions at m/z 328, 314 and 187 was determined by triplicate injection of each sample deriving from incubation with either natural water or with 12% ^2H_2O enrichment under both fed and fasted conditions.

Statistical analysis

The significance of the differences was determined by Student's t-test.

RESULTS AND DISCUSSION

The role of the liver in glucose homeostasis has been clearly established.[1] The liver can maintain the blood glucose level through two mechanisms: gluconeogenesis, via intermediate metabolites such as lactate and pyruvate, and glycogenolysis. Therefore, the use of hepatocytes to study modifications of glucose metabolism is unquestionable. Since the utility of stable isotopes in studies on glucose metabolism is also well known, we studied conditions of cell incubation in ^2H_2O in the attempt to set up a procedure to be used to establish the modulation of gluconeogenesis under various conditions and by drugs.

Since we were interested in establishing modifications of the fraction of glucose formed from lactate and pyruvate, the C-6/C-2 enrichment ratio was considered even though underestimation had been reported.[6] To obtain enrichments at the two carbon atoms, fragments at m/z 328, 314, 242, 212, 187 and 145 in the mass spectrum of aldonitrile pentaacetate of glucose [Fig. 2(A)] were first considered. Under the conditions used no interference was observed in the analysis of clusters of all selected ions by matrix components.

Enrichment at C-6 (E6)

Labeling at C-6 could be determined as the difference between deuterium enrichments of C-2, C-3, C-4, C-5 and C-6 (ME of ion at m/z 328; total enrichment) and those of C-2, C-3, C-4 and C-5 (ME of ion at m/z 212). Considering the low abundance of the former ion (Fig. 2), the possibility suggested by Guo *et al.*[5] of determining total enrichment as the sum of the ME of ions at m/z 145 (C-5 and C-6) and m/z 242 (C-2, C-3 and C-4) instead of enrichment of the ion at m/z 328 was considered. Concerning the signal at m/z 145, the non-homogeneity of the signal reported by Guo *et al.*[5] was also observed by us. In fact, assuming for the m/z 145 ion a structure including C-5 and C-6 of glucose, no signal at m/z 145 should be present in the spectrum of aldonitrile pentaacetate prepared from glucose containing two deuterium atoms at position 6. In contrast, in the spectrum of the derivative of 99.7% enriched [6,6-$^2H_2$]glucose [Fig. 2(B)] this ion was unexpectedly still present. As suggested by Guo *et al.*,[5] this type of interference does not exclude the use of the ion to evaluate glucose enrichment provided that the interference is considered to be constant and its presence is taken into consideration in the calculations.

Concerning the ion at m/z 242, no problem was described previously. However, we found that, in contrast to all other ions tested, the basal isotope ratios (mean $\pm$ SD, $n = 3$) determined for this ion in standard glucose (0.1439 ± 0.0013) and in glucose obtained from cells incubated in unlabeled medium (0.1480 ± 0.0015) were significantly higher than that expected on the basis of the natural enrichment of the fragment considered (0.1198). The non-unitary composition of the signal may be determined by analyzing the mass spectra of glucose labeled with deuterium at different positions. Nevertheless, the results reported by Guo *et al.*[5] suggest that commercially available deuterated standards[5] are not sufficiently pure to help in clarifying the problem. Based on the above considerations, the possibility of utilizing the sum of the ions at m/z 145 and 242 instead of the ion at m/z 328 to evaluate the total enrichment of glucose was discarded.

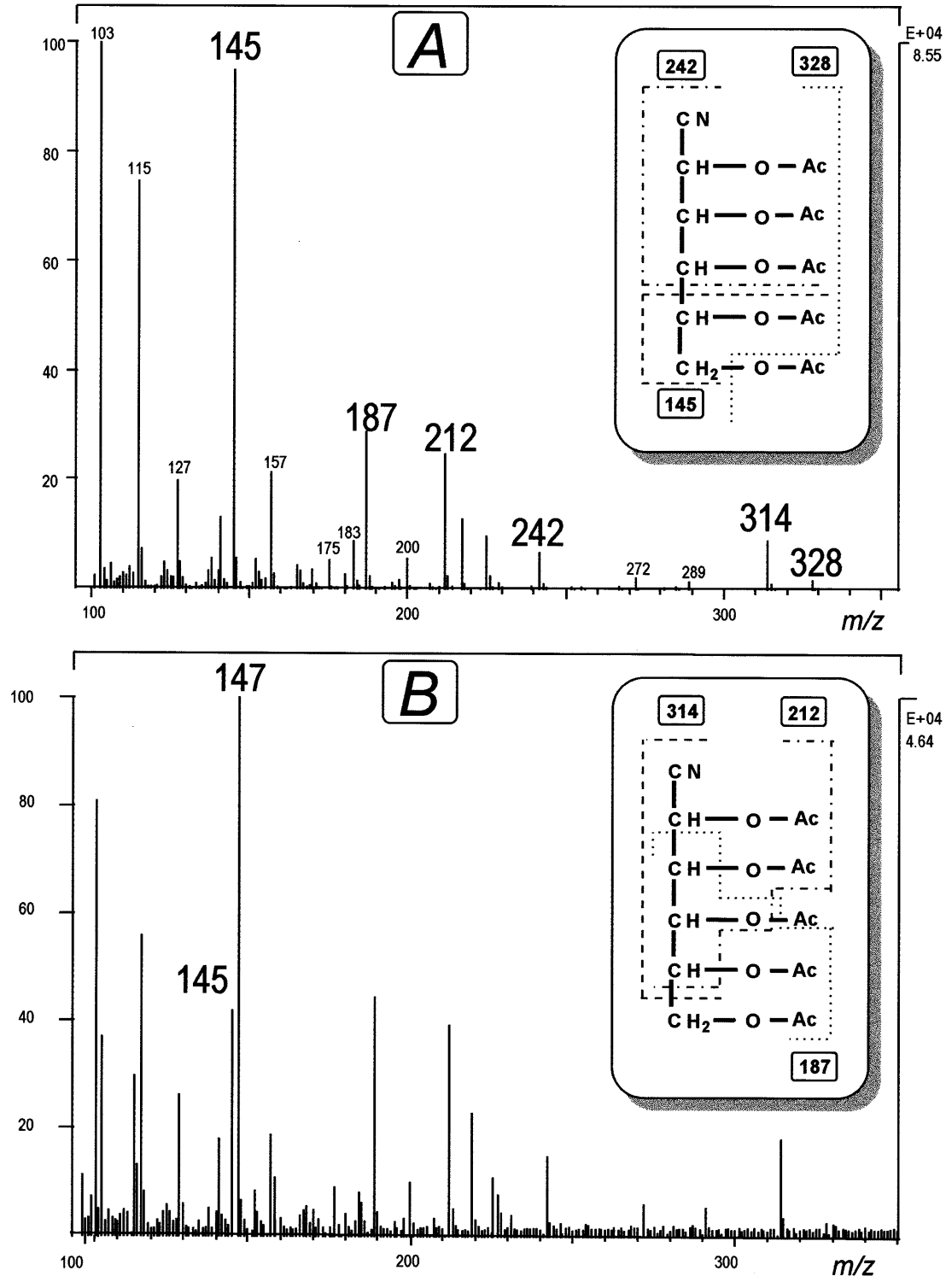

Figure 2. Mass spectra of aldonitrile pentaacetate of standard glucose (A) and [6,6-$^2H_2$]glucose (B).

Regarding the degree of isotope enrichment, conditions with both a high percentage of ^2H_2O (25% ^2H_2O) in the incubation medium and a high incorporation at C-6 (cells from fasted rats) were first considered. Results from the analysis of the deuterium distribution in fragments at *m/z* 328 and 212 in four incubation samples with the same cell preparation are given in Table 1. Isotopomers deriving from the incorporation of 1–4 deuterium atoms per molecule were readily detectable and were taken into account in the calculations. The standard deviations reported in Table 1 for the determination of MPE and ME indicate that the imprecision (RSD) was ~5%.

Conditions of higher substrate concentration that could increase the total glucose production were also tested in order to ensure conditions of higher sensitivity of the method as far as the evaluation of ME is concerned. Table 2 gives the ME values obtained with cell preparations from a fasted rat incubated with 25% ^2H_2O. At 20 mM substrate concentration enrichment

Table 1. Glucose enrichment obtained on incubating hepatocytes from a fasted rat with ^2H_2O[a]

m/z	MPE_1	MPE_2	MPE_3	MPE_4	ME
328	17.6 ± 0.7	12.8 ± 0.6	5.5 ± 0.9	1.1 ± 0.2	63.1 ± 2.9
212	20.2 ± 0.8	9.2 ± 0.4	1.9 ± 0.2	n.d.[b]	44.2 ± 1.9
187	19.2 ± 0.9	10.0 ± 0.5	2.9 ± 0.2	n.d.	47.7 ± 2.5

[a] Cells from a fasted rat were incubated with 25% ^2H_2O in the presence of 2 mM pyruvate as the substrate. Results are means ± SD of four incubations (10^6 cells per sample) of the same cell preparation. MPE and ME calculations are reported in the text.
[b] n.d. = Not detectable.

was found to be increased significantly whereas glucose production was unaltered with respect to the 2 mM concentration.

The possibility was also considered of reducing the number of isotopomers of each ion cluster to be detected that could result in both an increase in sensitivity and simplification of calculations. This was done under conditions of lower enrichment of water. Results of C-6 labeling with various enrichments of the medium with ^2H_2O are reported in Table 3. As expected, deuterium incorporation into glucose (ME) decreased with decrease in ^2H_2O in the medium (Table 3). Consequently, there was a different distribution of deuterium within the clusters with a decrease in the number of labeled species to be considered for the calculation of total deuterium (MPE). The precision of ME determination was not affected by the percentage of ^2H_2O in the medium. Enrichment at C-6 calculated as $ME_{328} - ME_{212}$ was 24.3, 14.5 and 6.9 at 25, 12 and 6% ^2H_2O, respectively.

In order to check the conditions corresponding to the lowest incorporation at C-6, hepatocytes of a fed rat were incubated with either 2 or 20 mM pyruvate and either 25 or 12% ^2H_2O. Using 25% of ^2H_2O, isotopomers containing up to three deuterium atoms were relevant and had to be recorded, whereas with 12% of ^2H_2O, isotopomers with a maximum of two deuterium atoms were relevant. Unexpectedly, under these conditions of low incorporation at C-6, negative values for E6 were obtained. Therefore, the possibility of using the ion at *m/z* 314 that contains the same glucose carbons as the ion at *m/z* 212 was tested. E6 calculated from the *m/z* 328 − 314 ions was around zero, as expected (Table

Table 2. Effect of substrate concentration on glucose enrichment at C-6 and C-2 with ^2H_2O obtained with cells from a fasted rat[a]

Substrate	ME *m/z* 328	ME *m/z* 212	E6 $ME_{328} - ME_{212}$	ME *m/z* 187	E2 $ME_{328} - ME_{187}$
2 mM pyruvate	63.1 ± 2.9	44.2 ± 1.9	18.9 ± 1.8	47.7 ± 2.5	15.4 ± 2.1
20 mM pyruvate	77.5 ± 0.8[b]	53.1 ± 1.3[b]	24.3 ± 1.0	57.4 ± 1.5[b]	20.1 ± 1.3

[a] Cells from a fasted rat were incubated with 25% ^2H_2O in the presence of 2 or 20 mM pyruvate as the substrate. Results are means ± SD of four incubations (10^6 cells per sample) of the same cell preparation. ME and E calculations are reported in the text.
[b] $P < 0.01$ *vs*. 2 mM pyruvate.

Table 3. Effect of % ^2H_2O in the incubation medium on the composition of the clusters considered[a]

m/z	^2H_2O (%)	MPE_1	MPE_2	MPE_3	MPE_4	ME
328	25	21.9	14.9	6.8	1.3	77.5
	12	20.5	6.9	2.4	n.d.[b]	41.4
	6	15.1	2.1	0.7	n.d.	19.3
212	25	24.8	11.1	2.0	n.d.	53.1
	12	19.6	3.7	n.d.	n.d.	27.0
	6	10.7	0.9	n.d.	n.d.	12.4
187	25	23.5	12.1	3.2	n.d.	57.4
	12	19.9	4.1	0.5	n.d.	29.6
	6	11.8	1.1	n.d.	n.d.	14.0

[a] Cells from a fasted rat were incubated with 25% ^2H_2O in the presence of 20 mM pyruvate as the substrate. Results are means of four incubations (10^6 cells per sample) of the sample cell preparation. MPE and ME calculations are reported in the text. The RSDs for the ME of all ions and conditions are below 5%.
[b] n.d. = Not detectable.

Table 4. Glucose enrichment obtained on incubating hepatocytes from fed rats with ^2H_2O[a]

^2H_2O (%)	Pyruvate (mM)	ME[b] *m/z* 328	ME[b] *m/z* 314	E6[c] ME_{328}–ME_{314}	ME[c] *m/z* 187	E2 ME_{328}–ME_{187}
25[d]	2	27.1 ± 1.4	25.2 ± 0.8	1.9 ± 0.6	16.5 ± 0.9	10.6 ± 0.9
25[d]	20	28.2 ± 1.3	26.6 ± 1.4	1.5 ± 1.2	18.1 ± 2.3	10.0 ± 1.7
12[d]	2	11.5 ± 0.2	10.9 ± 0.1	0.6 ± 0.1	6.9 ± 0.4	4.6 ± 0.4
12[e]	20	13.6 ± 1.1[d,f]	13.2 ± 0.9[d,f]	0.7 ± 0.6[d]	9.0 ± 0.6[d,f]	4.6 ± 0.7[d]
		15.5 ± 1.7	13.4 ± 1.6	2.1 ± 1.0	8.0 ± 1.2	7.5 ± 1.5
		19.6 ± 2.0	15.5 ± 1.1	4.0 ± 2.5	9.8 ± 0.6	9.8 ± 1.8

[a] Results are means ± SD of four incubations (10^6 cells per sample) of the same cell preparation.
[b] ME calculation is reported in the text.
[c] Enrichments (E) were calculated as $ME_{328} - ME_{314}$ (E6) and $ME_{328} - ME_{187}$ (E2).
[d] Cells from the same fed rat incubated with ^2H_2O and pyruvate as indicated.
[e] Cells from three fed rats incubated with 12% ^2H_2O and 20 mM pyruvate as indicated.
[f] $P < 0.01$ *vs.* 2 mM pyruvate with the same cell preparation.

4). These results suggest that also the ion at *m/z* 212 has a non-unitary composition. Guo *et al.*[5] did not describe problems using this ion in their calculations. However, it is worth noting that when gluconeogenesis and enrichment are not at the lowest level such as that occurring in fed rats, consistent results were also obtained by us using this ion. In fact, no difference was found calculating E6 either as *m/z* 328 − 212 or as *m/z* 328 − 314 for incubations with cells from fasted rats when the medium enrichment was 12% or higher (77 ± 12 *vs.* 72 ± 8 and 86 ± 7 *vs.* 83 ± 7 for 12 and 25% ^2H_2O, respectively).

Enrichment at C-2 (E2)

Labeling at C-2 was calculated as the difference between total enrichment (*m/z* 328) and enrichment at C-3, C-4, C-5 and C-6 (*m/z* 187). Under our conditions, no interferences were evident for the ion at *m/z* 187. The MPE and ME values for the ions tested are reported in Table 2. As described above for the other ions, high values of labeling were observed when incubation was performed in 25% ^2H_2O with cells obtained from a fasted animal. A higher substrate concentration resulted in the enhancement of both ME of the ion at *m/z* 187 and of E2 (Table 2). MPE values decreased on lowering the percentage of ^2H_2O in the incubation medium (Table 3). Nevertheless, the imprecision was low (< 5%) under all the conditions tested. Enrichment of the ion at *m/z* 187 was measurable even with a lower percentage of ^2H_2O and with cells from a fed rat (Table 4). It is worth noting that, as in the case of the fasted rat (Table 2), an increase in enrichment is obtained with increasing pyruvate concentration. However, in this case the increment is significant only with the lower enrichment of the medium.

Gluconeogenesis

In hepatocytes from fasted rats incubated with 20 mM pyruvate, the percentage of gluconeogenesis was in the range 60–66%. These observations were consistent with 67% gluconeogenesis calculated from data from *in vivo* experiments reported by Guo *et al.*[5] and with the 59–84% from experiments by Landau *et al.*[6] Moreover, Shalwitz *et al.*[13] demonstrated that gluconeogenesis was suppressed in fed as compared with fasted animals. This result is in good agreement with 7.3–3.7% found here for cells obtained from fed rats. No statistical differences were observed among the gluconeogenesis values obtained under the tested enrichment of water in the incubation medium: 60.8 ± 3.4, 61.7 ± 5.5 and 66.1 ± 5.1% for ^2H_2O at 25, 12 and 6%, respectively, for fasted rats and 7.2 ± 4.6 and 6.4 ± 5.0% for ^2H_2O at 25 and 12%, respectively, for fed rats. A level of 6% ^2H_2O for fed rats was considered to be too low to be detectable with acceptable imprecision.

Based on a previous observation[13] that species with only one deuterium may be representative of the enrichment, gluconeogenesis was calculated considering either mono-labeled or mono-labeled plus di-labeled species and the results were compared with those obtained considering all detectable deuterated species (Fig. 3). Non-significant differences were obtained for gluconeogenesis determined in 6, 12 and 25% deuterium-enriched medium both when all isotopormens of the cluster and when mono- plus di-labeled species were considered. In contrast, determination of E6/E2 considering only mono-labeled species gave erroneous results with both 12 and 25% enrichment. At low enrichment (6% ^2H_2O) mono-labeled species well represented the total enrichment, in agreement with the assumption introduced by Shalwitz *et al.*[13] in *in vivo* experiments. Nevertheless, this type of enrichment appeared to be too low to obtain consistent results under conditions of low gluconeogenesis. Based on the above considerations, the final conditions selected were incubation with 12% ^2H_2O, complete cluster evaluation and E6 and E2 calculation from *m/z* 328 − 314 and 328 − 187, respectively.

The absence of any isotope effect under these conditions was deduced from the glucose levels found in the medium of cells incubated either without ^2H_2O or with 12% enrichment of the medium. In fact, the glucose concentration (mg dl^{-1}) under the latter conditions (38.0 ± 3.5 ($n = 4$) and 9.1 ± 2.9 ($n = 3$) with fed and fasted rats, respectively) did not differ significantly from that of controls incubated in non-labeled medium (39.0 ± 4.6 ($n = 3$) and 5.7 ± 2.2 ($n = 4$) with fed and

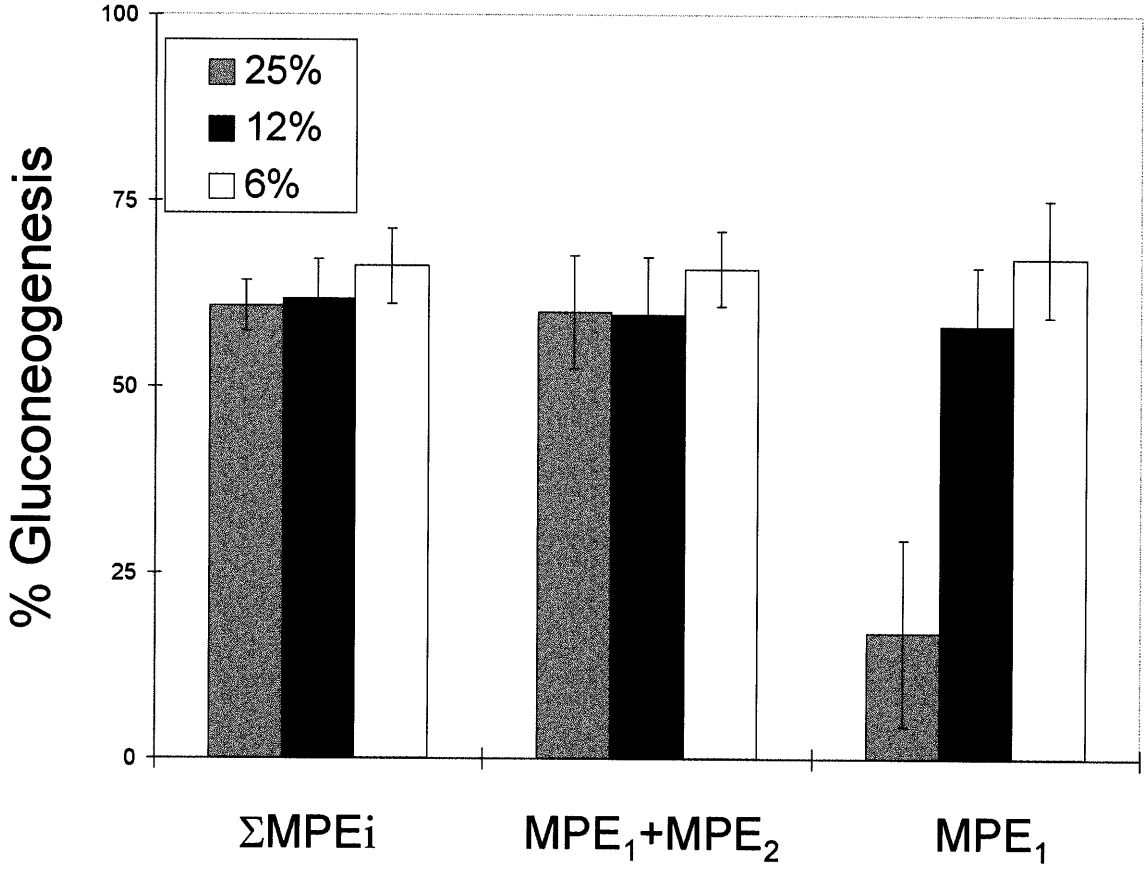

Figure 3. Gluconeogenesis determined at various ^2H_2O levels from all ions in the cluster (ΣMPE_1), mono- and di-labeled species ($MPE_1 + MPE_2$) or mono-labeled species (MPE_1). Data are means of four incubations of samples with cells obtained from a fasted rat.

fasted rats, respectively). This is in good agreement with data reported by Wals and Katz[14] indicating that isotope effects on glucose metabolism are detectable at about 20% enrichment and become significant at 40% ^2H_2O.

The intra-assay imprecision of the analytical method was also determined under the final selected conditions (Table 5). The RSD for all MR_n measurements was $<5\%$ with the exception of MR_3 for the *m/z* 328 cluster, probably attributable to the low intensity of the signal at *m/z* 331. As expected, the RSDs for ME values were not importantly affected by the RSDs of MR of multi-deuterated species and were found to be in the 1–8% range. Comparison of these results with those reported in the literature is difficult because, to our knowledge no systematic work has been described in this respect. Imprecision was reported by Shalwitz *et al.*[13] (RSD $\leq 2\%$) but they did not describe how their RSD values were obtained. Moreover, Landau *et al.*[6] reported RSDs relative to a standard sample of 5.5–9% for position 6 and 13–15% for the low enrichment of position 2 tested ($n = 9$).

Gluconeogenesis under these conditions was determined with hepatocytes obtained from fed and fasted

Table 5. Reproducibility (RSD, %) of enrichments for the ions considered

m/z	^2H_2O (%)	Condition	MR_1	MR_2	MR_3	CR_1	CR_2	CR_3	MPE_1	MPE_2	MPE_3	ME
328	0		0.3	2.9	10.5	—	—	—	—	—	—	—
	12	Fasted	0.9	2.6	9.7	1.7	5.6	26.8	1.5	5.3	26.1	4.1
	12	Fed	1.6	3.6	—	5.3	15.4	—	4.9	15.0	—	7.3
314	0		0.3	0.9	—	—	—	—	—	—	—	—
	12	Fasted	0.3	1.2	—	0.7	7.1	—	0.7	7.1	—	0.8
	12	Fed	0.5	0.8	—	2.1	4.1	—	2.0	4.0	—	1.7
187	0		1.4	4.7	—	—	—	—	—	—	—	—
	12	Fasted	2.6	2.3	—	3.7	2.7	—	3.0	2.1	—	2.8
	12	Fed	1.7	1.6	—	6.7	2.8	—	6.5	2.6	—	5.3

[a] Results are RSDs of three injections of the same sample.

Table 6. Gluconeogenesis evaluated in incubations of hepatocytes with ^2H_2O[a]

	Gluconeogenesis (%)	
	Fed rats[b]	Fasted rats[c]
	6.4	61.4*
	13.4	61.7
	18.6	58.1
		45.0
Mean ± SD	12.8 ± 6.1	56.6 ± 7.9

[a] Gluconeogenesis was calculated as reported in the text for ions m/z 328 − 314 (E6) and m/z 328 − 187 (E2). Results are means of four incubation samples (10^6 cells per sample) of the same cell preparation.
[b] Results obtained with cells from three fasted rats incubated with 12% ^2H_2O and 20 mM pyruvate.
[c] Results obtained with cells from one fasted rat (*) incubated with 25% ^2H_2O and 2 mM pyruvate and from three fed rats incubated with 12% ^2H_2O and 20 mM pyruvate.

rats (Table 6). Gluconeogenesis in fasted rats (56.6 ± 7.9%, $n = 4$) was consistent with the data reported by Guo *et al.*[5] *in vivo* (61–79%, $n = 4$) and by Shalwitz *et al.*[13] (45 ± 1%, $n = 3$). The latter authors reported gluconeogenesis under fed conditions *in vivo* to be 24 ± 1% ($n = 3$), which agrees well with the 14.5 ± 7.3% ($n = 3$) gluconeogenesis found in this work.

From all the reported results, it clearly appears that gluconeogenesis can be evaluated *in vitro* by liver cell incubation in ^2H_2O. Conditions allowing the evaluation of both very high and very low gluconeogenesis relative to total glucose production with good reproducibility imply monitoring clusters of three ions, at m/z 328, 314 and 187, and calculating the enrichment at C-6 from the difference of the ions at m/z 328 and 314 and the enrichment at C-2 from the difference of the ions at m/z 328 and 187. A level of 12% ^2H_2O is considered to be the optimum because it allows the number of isotopomers to be monitored to reduce thus increasing both the sensitivity and precision, and also avoiding isotope effects.

In conclusion, the present method represents a useful tool for estimating the contribution of gluconeogenesis to hepatic glucose production and to determining rates of gluconeogenesis *in vitro*.

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REFERENCES

1. R. R. Wolfe, in *Radioactive and Stable Isotope Tracers in Biomedicine*. Wiley-Liss, New York (1992).
2. A. L. Esterman, B. I. Cohen and N. B. Javitt, *J. Lipid Res.* **26**, 950 (1985).
3. J. K. Kelleher and T. M. Masterson, *Am. J. Physiol.* **262**, E118 (1992).
4. R. Rognstad, D. G. Clark and J. Katz, *Eur. J. Biochem.* **47**, 383 (1974).
5. Z. Guo, W. N. Lee, J. Katz and A. E. Bergner, *Anal. Biochem.* **204**, 273 (1992).
6. B. R. Landau, J. Wahren, V. Chandramouli, W. C. Schumann, K. Ekberg and S. C. Kalhan, *J. Clin. Invest.* **95**, 172 (1995).
7. B. R. Landau, J. Wahren, V. Chandramouli, W. C. Schumann, K. Ekberg and C. Kalhan, *J. Clin. Invest.* **98**, 378 (1996).
8. J. Katz, P. A. Wals, S. Golden and D. Rognstad, *Eur. J. Biochem.* **60**, 91 (1975).
9. J. W. Phillips, D. G. Clark, D. C. Henly and M. N. Berry, *Eur. J. Biochem.* **277**, 352 (1995).
10. P. O. Segle, in *Methods in Cell Biology*, edited by D. M. Prescott, p. 29. Academic Press, New York (1976).
11. F. Magni, R. Paroni, P. A. Bonini and M. Galli Kienle, *Clin. Chem.* **38**, 381 (1992).
12. F. Magni, L. Monti, P. Brambilla, R. Poma, G. Pozza and M. Galli Kienle, *J. Chromatogr.* **573**, 125 (1992)
13. R. A. Shalwitz, T. J. Beth, A.-M. K. MacLeod, S. J. Tucker and G. G. Rolison. *Am. J. Physiol.* **266**, E433 (1994).
14. P. A. Wals and J. Katz, *Int. J. Biochem.* **25**, 1561 (1993).
15. J. C. Garrison and R. C. Haynes, Jr, *J. Biol. Chem.* **248**, 5333, 1973.
16. S. R. Wagle, W. R. Ingebretsen, Jr, and L. Sampson, *Acta Diabet. Lat.* **123**, 185 (1975).