Measurement of pentose phosphate-pathway activity in a single incubation with [1,6-¹³C₂,6,6-²H₂]glucose

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The isotopically substituted molecule D-[1,6-¹³C₂,6,6-²H₂]glucose is introduced for measuring the relative activities of the pentose phosphate pathway (PPP) and glycolysis in a single incubation. PPP activity in cultured cells was determined by gas chromatography/mass spectrometric analysis of lactate produced by cells incubated with [1,6-¹³C₂,6,6-²H₂]glucose. Two other isotopes, [1,5,6-¹³C₃]glucose and [6-¹³C,1,6,6-²H₃]glucose, were not satisfactory for measurements of this activity. This method has four advantages over the traditional one in which ¹⁴CO₂ production from [1-¹⁴C]glucose and [6-¹⁴C]glucose is

compared: (1) repeated measurements can be made on a single set of cells, (2) only a single incubation is required, (3) extensive CO₂ production by Krebs-cycle activity does not interfere with the measurements and (4) it is not necessary to measure the amount of glucose consumed in order to calculate relative activities of the PPP and glycolysis. Preliminary observation indicates that rat brain PPP activity may be measured *in vivo* with [1,6-¹³C₂,6,6-²H₂]glucose when combined with microdialysis techniques.

INTRODUCTION

The oxidative branch of the pentose phosphate pathway (PPP) produces ribose 5-phosphate, CO₂ and NADPH from glucose 6-phosphate and NADP⁺ [1,2]. Ribose 5-phosphate can be used for nucleotide formation, or it can be converted by the non-oxidative branch of the PPP to fructose 6-phosphate and glyceraldehyde 3-phosphate. These latter two metabolites can then be converted into lactate by glycolytic enzymes, and fructose 6-phosphate can also recycle through the PPP. NADPH can be used for lipid synthesis and for regeneration of GSH from GSSG. Cellular GSH is important for protection from oxidative stress, as it is a substrate for glutathione peroxidase, which reduces H₂O₂ to water.

Activity of the PPP is commonly quantified by measuring ¹⁴CO₂ production from the C-1 of [1-¹⁴C]glucose; a parallel incubation with [6-¹⁴C]glucose is necessary to correct for ¹⁴CO₂ production from the citric acid cycle [1–4]. This method has four limitations: (1) only a single measurement can be made on each sample because the sample must be destroyed by acidification to release CO₂; (2) extensive Krebs-cycle activity can produce large amounts of ¹⁴CO₂ from both [1-¹⁴C]glucose and [6-¹⁴C]glucose, thus making it difficult to quantify low levels of PPP activity [5]; (3) two parallel incubations, with [1-¹⁴C]glucose and [6-¹⁴C]glucose, must be performed for each measurement, and intersample variability increases the uncertainty in the data; (4) calculations of PPP activity require measurement of the amount of glucose consumed [2–4].

The first two limitations can be overcome by measuring the

isotopic composition of triose phosphate derivatives such as lactate rather than CO₂ [1–3]. PPP activity can be measured by gas chromatographic/mass spectrometric (g.c./m.s.) analysis of lactate extruded from cultured cells after parallel incubations with [1-¹³C]glucose and [6-¹³C]glucose [6].

In this study, we have investigated the use of multiply labelled glucose, with distinguishable labels in the C-1 and C-6 positions, to avoid the need for parallel incubations. The lactate methyl groups derived from the C-1 and C-6 positions of these glucose isotopes can be distinguished by g.c./m.s., allowing the relative amounts of C-1 and C-6 in lactate to be quantified. Comparisons with the method utilizing [1-¹³C]glucose and [6-¹³C]glucose [6] indicate that [6-¹³C,1,6,6-²H₃]glucose and [1,5,6-¹³C₃]glucose are ineffective in quantifying PPP activity. These studies culminated in the development of the quadruply labelled isotope [1,6-¹³C₂,6,6-²H₂]glucose (Figure 1), which yielded accurate measurements of PPP activity in cultured glioma cells. Preliminary data show the potential for measuring the activity of the PPP in rat brain *in vivo* with [1,6-¹³C₂,6,6-²H₂]glucose when combined with microdialysis techniques [7].

EXPERIMENTAL

Synthesis of glucose isotopes

All isotopically substituted D-glucose molecules were purchased from Omicron Biochemicals (South Bend, IN, U.S.A.), and their atom % enrichments were determined using high-resolution ¹H- and ¹³C-n.m.r. spectroscopy and g.c./m.s. The synthesis of [1,5,6-¹³C₃]glucose has been reported recently [8].

Abbreviations used: g.c./m.s., gas chromatography/mass spectrometry; KRB, Krebs–Ringer bicarbonate buffer; PMS, phenazine methosulphate; PPP, pentose phosphate pathway; [0]CO₂, CO₂ containing neither C-1 nor C-6 of the original labelled glucose; [C1]CO₂, CO₂ containing C-1 of the original labelled glucose; T, one-third of the number of mol of CO₂ produced by the PPP per mol of glucose metabolized to lactate and/or CO₂; [C1]lactate, lactate with a methyl group derived from C-1 of labelled glucose; [C6]lactate, lactate with a methyl group derived from C-6 of labelled glucose; [0]lactate, lactate with a methyl group derived from neither C-1 nor C-6 of labelled glucose; E, the number of mol of lactate produced from endogenous sources for each mol of labelled glucose converted into lactate and/or CO₂ by glycolysis and PPP; 1- κ_1 and 1- κ_2 , the fraction of ²H atoms lost from ²H₁- and ²H₂-labelled trioses; 1- μ , the additional fraction of ²H lost from C-1 of glucose, presumably as a result of phosphomannose isomerase activity; P, the fraction of glucose metabolized to lactate that loses C-1 as CO₂ by PPP activity; Y, the fraction of fructose 6-phosphate from the PPP that returns to glucose 6-phosphate before being metabolized further by either the PPP or glycolysis; L_H, fractional ²H enrichment; L_C, fractional ¹³C enrichment; Λ_C , (1-L_C)/L_C.

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Figure 1 Biochemical rationale for the use of [1,6- $^{13}\text{C}_2$,6,6- $^2\text{H}_2$]glucose to quantify PPP activity

Metabolism via glycolysis will produce a 1:1 mixture of [3- ^{13}C ,3,3- $^2\text{H}_2$]lactate/[3- ^{13}C]lactate, whereas degradation through the PPP will produce unlabelled lactate and [3- ^{13}C ,3,3- $^2\text{H}_2$]lactate.

Cell culture conditions

Rat 9L glioma cells were grown to confluence in 24-well flat-bottomed sterile tissue culture plates in minimum essential medium containing 10% fetal bovine serum and antibiotics in a 5% CO_2 /air incubator at 37°C. Immediately before the incubation experiments, flasks were rinsed three times with Krebs-

Ringer bicarbonate buffer (KRB), pH 7.4. Four replicate wells were used for each experimental condition. Each well was incubated for 1 h with 0.5 ml of KRB supplemented with an isotopically substituted glucose (5.5 or 2.8 mM) under the various conditions summarized in Table 1. After the incubation, the buffer was removed and lyophilized for g.c./m.s. analysis.

Cultured-cell PPP activities from incubations with the newly synthesized glucose molecules, [6- ^{13}C ,1,6,6- $^2\text{H}_2$]glucose, [1,5,6- $^{13}\text{C}_3$]glucose and [1,6- $^{13}\text{C}_2$,6,6- $^2\text{H}_2$]glucose were compared with the PPP activity obtained from parallel incubations with [1- ^{13}C]glucose and [6- ^{13}C]glucose [6]. The following experiments were conducted to evaluate these molecules for quantification of PPP activity. All incubations in Experiments 1A–1D were carried out in parallel whereas Experiments 2 and 3 were performed at a later time.

Experiment 1A

Glioma cells were incubated with KRB containing [1- ^{13}C]glucose or [6- ^{13}C]glucose with and without 5 μM phenazine methosulphate (PMS), which stimulates PPP activity by oxidizing NADPH to NADP $^+$ [4].

Experiment 1B

Glioma cells were incubated with [6- ^{13}C ,1,6,6- $^2\text{H}_2$]glucose. Parallel incubations with [1- ^{13}C ,1- ^2H]glucose and [6- ^{13}C ,6,6- $^2\text{H}_2$]glucose were also performed to investigate the extent of deuterium loss from the C-1 and C-6 positions of glucose.

Experiment 1C

Glioma cells were incubated with [1,5,6- $^{13}\text{C}_3$]glucose and [5- ^{13}C]glucose to check for selective loss of the C-5 carbon atom. In a subsequent study, the ability of [1,5,6- $^{13}\text{C}_3$]glucose to measure changes in PPP activity was investigated by addition of 5 μM PMS.

Experiment 1D

Glioma cells were incubated with 5.5 and 2.8 mM [1,6- $^{13}\text{C}_2$,6,6- $^2\text{H}_2$]glucose, alone or with 5 μM PMS.

Table 1 Measurement of PPP activity under various conditions in cultured 9L glioma cells

Cells were incubated as described in the Experimental sections. PPP activity, P , is expressed as mean $\pm$ S.D. from four incubations for each condition as indicated.

Glucose isotope(s)	Conditions	P (%)
Experiment 1A		
[1- ^{13}C]Glc and [6- ^{13}C]Glc	5.5 mM	7.1 $\pm$ 2.5
[1- ^{13}C]Glc and [6- ^{13}C]Glc	5.5 mM + 5 μM PMS	37.2 $\pm$ 2.9
Experiment 1B		
[6- ^{13}C ,1,6,6- $^2\text{H}_2$]Glc	5.5 mM	28.9 $\pm$ 2.2
Experiment 1C		
[1,5,6- $^{13}\text{C}_3$]Glc	5.5 mM	-8.9 $\pm$ 2.6
[1- ^{13}C]Glc and [5- ^{13}C]Glc	5.5 mM	9.4 $\pm$ 2.3
Experiment 1D		
[1,6- $^{13}\text{C}_2$,6,6- $^2\text{H}_2$]Glc	5.5 mM	9.4 $\pm$ 2.1
[1,6- $^{13}\text{C}_2$,6,6- $^2\text{H}_2$]Glc	2.8 mM	9.4 $\pm$ 2.0
[1,6- $^{13}\text{C}_2$,6,6- $^2\text{H}_2$]Glc	2.8 mM + 5 μM PMS	30.6 $\pm$ 1.9
Experiment 2		
[1,6- $^{13}\text{C}_2$,6,6- $^2\text{H}_2$]Glc	5.5 mM	6.4 $\pm$ 0.3
[1,6- $^{13}\text{C}_2$,6,6- $^2\text{H}_2$]Glc	4.4 mM (80%) + 1.1 mM Glc (20%)	5.8 $\pm$ 0.7
[1,6- $^{13}\text{C}_2$,6,6- $^2\text{H}_2$]Glc	5.5 mM + 5 μM PMS	48.5 $\pm$ 1.8
[1,6- $^{13}\text{C}_2$,6,6- $^2\text{H}_2$]Glc	4.4 mM (80%) + 1.1 mM Glc (20%) + 5 μM PMS	41.8 $\pm$ 3.6

Experiment 2

In order to establish that unlabelled lactate precursors would not interfere with PPP measurements using [1,6- $^{13}\text{C}_2$,6,6- $^2\text{H}_2$]glucose, 9L glioma cells were incubated with a KRB solution containing 4.4 mM [1,6- $^{13}\text{C}_2$,6,6- $^2\text{H}_2$]glucose (80 %) and 1.1 mM unlabelled glucose (20 %) with and without PMS.

Experiment 3

To demonstrate the ability to make repetitive measurements on a single set of cells, KRB (0.5 ml) containing 5.5 mM [1,6- $^{13}\text{C}_2$,6,6- $^2\text{H}_2$]glucose was added to and pipetted from eight wells every hour during a 5 h time period. The conditions for all the wells were identical except that, in four of them, cells were exposed to 5 μM PMS during the third 1 h time interval in order to stimulate PPP activity. After each 1 h incubation period, the buffer was removed and lyophilized for g.c./m.s. analysis.

G.c./m.s.

For g.c./m.s. analysis, dried samples were converted into their trimethylsilyl (Me_3Si) derivatives by addition of 50 μl of pyridine and 50 μl of bis(trimethylsilyl)trifluoroacetamide with 1 % trimethylchlorosilane (Alltech Associates Inc., Deerfield, IL, U.S.A.) and heated to 50 $^\circ\text{C}$ for 15 min before injection as previously described [9]. Mass spectra were acquired on a Hewlett-Packard g.c./m.s. (Model 5890/5971) in the selected ion-monitoring mode on the m/z 219 to m/z 222 ions. These ions correspond to the loss of a methyl group from the Me_3Si derivative of lactic acid at an ionization energy of 70 eV. In order to correct for the 'tail' produced by the isotopic composition of the Me_3Si lactate derivative, the expected isotopic composition of this derivative was calculated from standard values of natural isotope abundance [10]: 98.9 % ^{12}C , 1.1 % ^{13}C , 99.985 % ^1H , 0.015 % ^2H , 99.76 % ^{16}O , 0.04 % ^{17}O , 0.2 % ^{18}O , 92.23 % ^{28}Si , 4.67 % ^{29}Si and 3.10 % ^{30}Si . Adjustments were made for positions that contained a known ^{13}C or ^2H atom derived from labelled glucose, and the resulting intensities in the $m+0$ – $m+3$ peaks are shown in Table 2. Lactate standards were checked routinely in order to evaluate the g.c./m.s. performance.

A HP-5 fused-silica column (30 m $\times$ 0.25 mm internal diameter) with a 0.25 μm film thickness (Hewlett-Packard, Wilmington, DE, U.S.A.) was used for gas chromatography with helium as the carrier gas. Typically, 12–24 samples were derivatized at one time and placed in an automatic liquid-sample injection system (H.P. model 7673) for overnight g.c./m.s. analysis. Derivatized samples (1 μl) were injected into the column through the standard split/splitless injection port operated in the split mode (30:1) at 270 $^\circ\text{C}$. The column temperature was programmed for 0.5 min at 60 $^\circ\text{C}$ followed by a temperature ramp at 15 $^\circ\text{C}/\text{min}$ to 140 $^\circ\text{C}$. The temperature was then increased to 280 $^\circ\text{C}$ at 30 $^\circ\text{C}/\text{min}$ and held for 2 min. G.c./m.s. quantification of the m/z 219– m/z 222 ions was performed on triplicate injections of each individual sample. PPP activity was calculated for each injection and the average value of the PPP activity for each sample was used in error analysis. All data are expressed as means $\pm$ S.D. of multiple samples.

Metabolic model: definition of PPP activity

Total PPP activity can be calculated by measuring (1) $G_{\text{met.}}$, the total amount of glucose converted into lactate and/or CO_2 and (2) P , the fraction of glucose converted into lactate that lost C-1 as it passed through the PPP. Lactate production can be measured in several ways; if Krebs-cycle activity is low, this is a

Table 2 Correction factors for g.c./m.s. data of lactate with specific isotope compositions

Calculations of the relative intensities of the peaks of the mass spectrum of the trimethylsilyl derivative of lactate with isotopically substituted atoms were based on standard values of natural isotope abundance [10]. In the absence of any isotopic substitution, $n = 0$ corresponds to m/z 219 ($\text{M}^+ - \text{CH}_3$).

Isotope	Relative amount in the mass + n peak				
	$n = 0$	$n = 1$	$n = 2$	$n = 3$	$n = 4$
$^{13}\text{C}_0^2\text{H}_0$	100	19.43	8.91	1.15	0.24
$^{13}\text{C}_0^2\text{H}_1$	100	19.42	8.90	1.15	0.24
$^{13}\text{C}_0^2\text{H}_2$	100	19.40	8.90	1.15	0.24
$^{13}\text{C}_1^2\text{H}_0$	100	18.32	8.70	1.06	0.23
$^{13}\text{C}_1^2\text{H}_1$	100	18.30	8.70	1.06	0.23
$^{13}\text{C}_1^2\text{H}_2$	100	18.29	8.70	1.05	0.23
$^{13}\text{C}_2^2\text{H}_0$	100	17.21	8.51	0.96	0.21

Table 3 Labelled and unlabelled metabolic products from isotopically substituted glucose molecules

Values represent lactate equivalents of product (1 mol of lactate or 3 mol of CO_2) per mol of labelled glucose degraded to CO_2 and lactate by glycolysis and the PPP. Terms and symbols are defined in the text.

Product	Amount produced
[C1] CO_2	$P/3$
[0] CO_2	$(2P/3)[PY/(3-2PY)]$
[0]lactate	$(2P/3)[(3-3PY)/(3-2PY)] + E$
[C1]lactate	$1 - P$
[C6]lactate	1

Table 4 Formulae for analysis of experimental data

The parameters R_1 and R_6 are measured from experimental data and the formulae given under 'Description'. The parameters P , T , r_6 and E are calculated from the values of R_1 and R_6 and the formulae given under 'Equivalent formula'.

Parameter	Description	Equivalent formula
R_1	$[\text{C1}] \text{ lactate} / \{ [\text{O}] + [\text{C1}] + [\text{C6}] \} \text{ lactate}$	$(1 - P) / (2 + E - T)$
R_6	$[\text{C6}] \text{ lactate} / \{ [\text{O}] + [\text{C1}] + [\text{C6}] \} \text{ lactate}$	$1 / (2 + E - T)$
P	$1 - \{ [\text{C1}] \text{ lactate} / [\text{C6}] \text{ lactate} \}$	$1 - R_1 / R_6$
T	$[0] \text{CO}_2 + [\text{C1}] \text{CO}_2$	$P / (3 - 2PY)$
r_6	$\{ [\text{O}] + [\text{C1}] \} \text{ lactate} / [\text{C6}] \text{ lactate}$	$1 + E - T$
E	Endogenous lactate	$r_6 + T - 1$

good estimate of $G_{\text{met.}}$. In this work, we present a new way to measure P , which corresponds to S or $1 - \gamma$ of Katz et al. [3] or $F_{\text{ent.}}$ of Larrabee [11]. The parameter P includes the action of glucose 6-phosphate dehydrogenase on only the original labelled glucose, not on glucose 6-phosphate derived by 'recycling' from fructose 6-phosphate produced by the PPP. The effects of recycling are included in the parameter T , which equals one-third of the number of mol of CO_2 produced by the PPP per mol of glucose metabolized to lactate and/or CO_2 . T is represented in Table 3 by the sum $[0] \text{CO}_2$ (CO_2 containing neither C-1 nor C-6 of the original labelled glucose) + $[\text{C1}] \text{CO}_2$ (CO_2 containing C-1 of the original labelled glucose). We report measured values of P ; T can be estimated from the formula in Table 4. If $G_{\text{met.}}$ is also measured, the product $TG_{\text{met.}}$ measures total flux through the

Table 5 Labelled lactate molecules derived from different isotopically substituted glucose

The indicated products do not include possible loss of ^2H as described in the text. This assumes metabolism through glycolysis and that labels are not scrambled by metabolism beyond pyruvate or lactate.

Label in glucose	Label in [C1]lactate	Label in [C6]lactate
1- ^{13}C	3- ^{13}C	None
6- ^{13}C	None	3- ^{13}C
1,5,6- $^{13}\text{C}_3$	3- ^{13}C	2,3- $^{13}\text{C}_2$
1- ^{13}C ,1- ^2H	3- ^{13}C ,3- ^2H	None
6- ^{13}C ,6,6- $^2\text{H}_2$	None	3- ^{13}C ,3,3- $^2\text{H}_2$
6- ^{13}C ,1,6,6- $^2\text{H}_3$	3- ^2H	3- ^{13}C ,3,3- $^2\text{H}_2$
1,6- $^{13}\text{C}_2$,6,6- $^2\text{H}_2$	3- ^{13}C	3- ^{13}C ,3,3- $^2\text{H}_2$

PPP. The values of P and T would differ by a factor of exactly 3 if recycling did not occur, and the factor is slightly less than 3 if P is small. Recycling of PPP products into the PPP is represented by the parameter Y , which is the mol fraction of fructose 6-phosphate that is converted into glucose 6-phosphate, and $1 - Y$ is the mol fraction of fructose 6-phosphate that is converted (irreversibly) into fructose 1,6-bisphosphate. The ratio $Y/(1 - Y)$ therefore equals the ratio of the two reaction rates $[\text{Fru6P} \rightarrow \text{Glc6P}]/[\text{Fru6P} \rightarrow \text{Fru}(1,6)\text{P}_2]$ catalysed by phosphoglucose isomerase and phosphofructokinase respectively.

Metabolic model: PPP calculation

The model utilized for PPP calculation is similar to that of Landau et al. [12], whose formulae can be converted into our formulae by the following substitutions: $E_{-h}/(1 - PC) = Y/(1 - Y)$; $G_2 = L_{C2}$; $f = V_3 = 0$; $PC = T$.

Calculation of PPP activity in cells or tissues from the metabolism of glucose labelled in the C-1 and/or C-6 positions by n.m.r. spectroscopy [9] or by g.c./m.s. [6,9] depends on the ability to detect and distinguish between labelled and unlabelled lactate. The lactate methyl group will be derived from the C-1 of the labelled glucose ([C1]lactate), the C-6 of labelled glucose ([C6]lactate) or neither ([0]lactate). The relative amounts of labelled and unlabelled CO_2 and lactate, expressed as lactate equivalents, are shown as a function of P and Y in Table 3. The lactate isotopomers formed from the labelled glucoses utilized in the present study are summarized in Table 5.

Formulae for determination of P , T and E [the amount of lactate produced from unlabelled (endogenous) precursors] from experimental data are shown in Table 4. The value of P is determined directly from either R_1 and R_6 measured in separate experiments or [C1]lactate and [C6]lactate measured in a single experiment. Calculation of T requires an estimate of Y , which is probably large [13] but must be less than unity. The limits on T are $P/3 \leq T \leq P/(3 - 2P)$. The value of E can be calculated from T and the ratio $r_6 = \{[0]\text{lactate} + [\text{C1}]\text{lactate}\}/[\text{C6}]\text{lactate}$.

More complex models

A more complex model like that of Landau and Bartsch [14], incorporating synthesis of glycogen and galactose, removal of pentose phosphates for nucleotide synthesis, removal of dihydroxyacetone phosphate for synthesis of phosphoglycerides and triacylglycerols (via *sn*-glycerol 3-phosphate) and numerous exchange reactions is not necessary in most cases, although it

may be necessary in special cases such as liver, adipose tissue [3] and lactating mammary gland. If certain reversible reactions are not quite in equilibrium, PPP activity may be slightly underestimated in the presence of glycogen, galactose and nucleotide synthesis, and slightly overestimated in the presence of lipid synthesis.

Assumptions and conventions

In all glucose molecules with two or more labels in one position (for example, $[6-^{13}\text{C}, 6,6-^2\text{H}_2]$ or $[1-^{13}\text{C}, 1-^2\text{H}]$), it is assumed that fractional enrichment in each label is independent of enrichment in any other label(s) in the same position, but the two ^2H atoms on C-6 of glucose may have different fractional enrichments. If enrichment is not independent, the formulae must be modified to reflect the actual isotope distribution. It is not necessary that C-1 labels be independent of C-6 labels. For example, $[6-^{13}\text{C}, 1,6,6-^2\text{H}_3]\text{glucose}$ could be replaced by a mixture of $[1-^2\text{H}]\text{glucose}$ and $[6-^{13}\text{C}, 6,6-^2\text{H}_2]\text{glucose}$, but not by a mixture of $[6-^{13}\text{C}]\text{glucose}$ and $[1,6,6-^2\text{H}_3]\text{glucose}$.

Lactate isotopes are often represented by a shorthand notation, for example, $^2\text{H}_2^{13}\text{C} = [3-^{13}\text{C}, 3,3-^2\text{H}_2]\text{lactate}$. Initial isotope enrichments are represented by, for example, $L_{\text{H6}} =$ enrichment of ^2H attached to C-6 of glucose. Terms of the form $(1 - L_c)/L_c$ appear frequently and will be abbreviated as $\Lambda_c = (1 - L_c)/L_c$.

In the following formulae, it is assumed that carbon labels are not lost (except by the PPP), although ^2H may be exchanged for ^1H . If ^2H and ^{13}C labels are independently distributed, then the amount of $[3-^{13}\text{C}, 3-^2\text{H}_n]\text{lactate}$ can be calculated from the amount of $[3-^{13}\text{C}, 3-^2\text{H}_n]\text{lactate}$ with the equation:

$$^2\text{H}_n^{13}\text{C} = (^2\text{H}_n^{13}\text{C})\Lambda_c \quad (1)$$

where Λ_c is defined above and L_c is the fractional enrichment of ^{13}C in the position of interest.

After the g.c./m.s. peaks have been resolved into individual isotope components, the values of P , T and E may be calculated from the formulae in Table 4. Although the formulae may appear lengthy, the calculations are easily performed using standard spreadsheet software on a personal computer. Detailed calculations will be shown for $[1-^{13}\text{C}]\text{glucose} + [6-^{13}\text{C}]\text{glucose}$ and for $[1,6-^{13}\text{C}_2, 6,6-^2\text{H}_2]\text{glucose}$. Similar calculations were performed for the other isotopically labelled glucose molecules.

Loss of ^2H labels

The use of an ^2H label introduces a new factor: the ^2H may be removed by exchange with water. For example, the loss of ^2H from the methyl group of pyruvate may occur non-enzymically, or it may be catalysed by pyruvate kinase [15] and possibly other enzymes. The rates of ^2H loss from $^2\text{H}_1$ - and $^2\text{H}_2$ -labelled trioses may be different because of secondary isotope effects [16]. For each ^2H atom, κ_1 and κ_2 are the probabilities that the ^2H atom is retained in a $^2\text{H}_1$ or $^2\text{H}_2$ -labelled triose respectively, when the final lactate molecule is observed. The probability of an ^2H atom being lost is therefore $1 - \kappa_1$ or $1 - \kappa_2$. If one ^2H is lost from an $^2\text{H}_2$ -labelled triose, the remaining ^2H atom has a probability κ_1 of being retained. We have used $\kappa_1 = \kappa_2$ for all our calculations, although typical isotope effects would result in a 10–15% greater loss of ^2H in $^2\text{H}_1$ -labelled trioses [16], or $R_{12} \equiv (1 - \kappa_1)/(1 - \kappa_2) \approx 1.1$. Our observed values of $1 - \kappa_2 \approx 0.10$, or $\kappa_2 \approx 0.90$, would mean $1 - \kappa_1 \approx 0.11$, or $\kappa_1 \approx 0.89$.

Phosphomannose isomerase, however, can catalyse an exchange of the ^2H label initially on C-1 of glucose via $\text{Fru6P} \leftrightarrow \text{Man6P}$ without affecting any ^2H attached to C-6 of glucose [16,17]. We refer to this exchange as $1 - \mu$, where $1 - \mu$ is the

Table 6 Isotope enrichment in lactate produced from [1,6-¹³C₂,6,6-²H₂]-glucose

The amount of lactate with each isotopic composition reflects incomplete fractional enrichment and possible metabolic ²H loss.

<i>m/z</i>	isotope	Amount in lactate
From C-6 of glucose		
222	² H ₂ ¹³ C	($\kappa_2 L_{H6a}$)($\kappa_2 L_{H6b}$)/ L_{C6}
221	² H ₂ ¹² C	($\kappa_2 L_{H6a}$)($\kappa_2 L_{H6b}$)(1 - L_{C6})
221	² H ₁ ¹³ C	[$\kappa_1 L_{H6a}(1 - \kappa_2 L_{H6b}) + \kappa_1 L_{H6b}(1 - \kappa_2 L_{H6a})$ + $L_{H6a} L_{H6b}(1 - \kappa_2)(\kappa_2 - \kappa_1)$]/ L_{C6}
220	² H ₁ ¹² C	$\kappa_1 L_{H6a}(1 - \kappa_2 L_{H6b}) + \kappa_1 L_{H6b}(1 - \kappa_2 L_{H6a})$ + $L_{H6a} L_{H6b}(1 - \kappa_2)(\kappa_2 - \kappa_1)$ (1 - L_{C6})
220	² H ₀ ¹³ C	[(1 - $\kappa_1 L_{H6a}$)(1 - $\kappa_1 L_{H6b}) - L_{H6a} L_{H6b}(1 - \kappa_1)(\kappa_2 - \kappa_1)]/L_{C6}$
219	² H ₀ ¹² C	[(1 - $\kappa_1 L_{H6a}$)(1 - $\kappa_1 L_{H6b}) - L_{H6a} L_{H6b}(1 - \kappa_1)(\kappa_2 - \kappa_1)](1 - L_{C6})$
From C-1 of glucose		
220	² H ₀ ¹³ C	L_{C1}
219	² H ₀ ¹² C	1 - L_{C1}

probability that an individual ²H atom will be lost in a phosphomannose isomerase-catalysed reaction. The parameters κ_2 and μ can be determined by g.c./m.s. analysis after incubations with [6-¹³C,6,6-²H₂]glucose and [1-¹³C,1-²H]glucose.

When glucose contains two geminal ²H atoms in the C-6 position, one or both of these may be missing in the original labelled glucose or may be lost during production of lactate. If the absence or loss of ²H in each site is independent of the other site, then the relative amounts of ²H₂-, ²H₁- and ²H₀-labelled molecules are shown in Table 6. In these equations L_{H6a} and L_{H6b} are the mol fractions of ²H attached to C-6 of glucose, and 1 - κ_1 and 1 - κ_2 represent loss of ²H atoms from ²H₁- and ²H₂-labelled trioses respectively, as described above.

The value of κ_2 can be estimated as follows:

$$S_6 \equiv (^{2}\text{H}_1^{13}\text{C})/(^{2}\text{H}_2^{13}\text{C}) \quad (2)$$

$$\kappa_2 = \frac{\kappa_{12}(L_{H6a} + L_{H6b}) + L_{H6a}L_{H6b}(1 - \kappa_{12})}{L_{H6a}L_{H6b}(S_6 + 1 + \kappa_{12})} \quad (3)$$

The parameter $\kappa_{12} = \kappa_1/\kappa_2$ can be set equal to unity (no isotope effect) for an initial calculation. To include isotope effects, the estimate of κ_2 and the estimated isotope effect, $R_{12} = (1 - \kappa_1)/(1 - \kappa_2) \approx 1.1$, can be used to estimate κ_1 and hence κ_{12} :

$$\kappa_1 = 1 - R_{12}(1 - \kappa_2) \quad (4)$$

The estimate of κ_2 can be refined by repeating the calculation with this new value of κ_{12} . The amount of lactate that has lost two ²H atoms can be calculated from the amounts that lost no ²H atoms or one ²H atom:

$$\begin{aligned} (^{2}\text{H}_0) = \\ \frac{(^{2}\text{H}_2)[(1 - \kappa_1 L_{H6a})(1 - \kappa_1 L_{H6b}) - L_{H6a}L_{H6b}(1 - \kappa_1)(\kappa_2 - \kappa_1)]}{(\kappa_2 L_{H6a})(\kappa_2 L_{H6b})} \end{aligned} \quad (5)$$

The loss of ²H from C-1 of glucose can be calculated from the following formulae, where L_{H1} is the mol fraction of ²H in glucose, 1 - κ_1 is the mol fraction of ²H that is lost from triose derivatives, and 1 - μ is the mol fraction of ²H that is lost from C-1 of glucose before formation of triose derivatives, presumably in reactions catalysed by phosphomannose isomerase.

$$S_1 \equiv (^{2}\text{H}^{13}\text{C})/(^{2}\text{H}^{13}\text{C} + ^{1}\text{H}^{13}\text{C}) = \mu \kappa_1 L_{H1} \quad (6)$$

$$\mu \kappa_1 = S_1/L_{H1} \quad (7)$$

If κ_1 is known, μ can be calculated.

Measurements with [1-¹³C]glucose and [6-¹³C]glucose

In parallel incubations with [1-¹³C]glucose and [6-¹³C]glucose, the *m/z* 219 peak corresponds to unlabelled lactate and the *m/z* 220 peak corresponds to [3-¹³C]lactate. With [1-¹³C]glucose,

$$R_1 = (m/z\ 220)/(m/z\ 219 + m/z\ 220)L_{C1} \quad (8)$$

With [6-¹³C]glucose,

$$R_6 = (m/z\ 220)/(m/z\ 219 + m/z\ 220)L_{C6} \quad (9)$$

$$r_6 = L_{C6}[(m/z\ 219) - (m/z\ 220)\Lambda_{C6}]/(m/z\ 220) \quad (10)$$

The values of *P*, *T* and *E* can be calculated from Table 4.

Measurements with [1,6-¹³C₂,6,6-²H₂]glucose

Metabolism of [1,6-¹³C₂,6,6-²H₂]glucose will yield six distinct isotopomers of lactate (Table 6). Six isotopomers are derived from C-6 of glucose:

$$^2\text{H}_2^{13}\text{C} = m/z\ 222 \quad (11)$$

$$^2\text{H}_2^{12}\text{C} = (m/z\ 222)\Lambda_{C6} \quad (12)$$

$$^2\text{H}_1^{13}\text{C} = (m/z\ 221) - ^2\text{H}_2^{12}\text{C} \quad (13)$$

$$^2\text{H}_1^{12}\text{C}[C6] = (^2\text{H}_1^{13}\text{C})\Lambda_{C6} \quad (14)$$

The values of κ_2 and κ_1 can be calculated from eqns. (3) and (4).

$$^2\text{H}_0^{13}\text{C}[C6] =$$

$$\frac{^2\text{H}_2^{13}\text{C}[(1 - \kappa_1 L_{H6a})(1 - \kappa_1 L_{H6b}) - L_{H6a}L_{H6b}(1 - \kappa_1)(\kappa_2 - \kappa_1)]}{(\kappa_2 L_{H6a})(\kappa_2 L_{H6b})} \quad (15)$$

$$^2\text{H}_0^{12}\text{C}[C6] = (^2\text{H}_0^{13}\text{C}[C6])\Lambda_{C6} \quad (16)$$

Two isotopomers come from C-1 of glucose:

$$^2\text{H}_0^{13}\text{C}[C1] = (m/z\ 220) - ^2\text{H}_1^{12}\text{C} - ^2\text{H}_0^{13}\text{C}[C6] \quad (17)$$

$$^2\text{H}_0^{12}\text{C}[C1] = (^2\text{H}_0^{13}\text{C}[C1])\Lambda_{C1} \quad (18)$$

To determine *P*, the isotopomers in eqns. (11)–(16) should be added to yield total [C6]lactate and the isotopomers in eqns. (17) and (18) should be added to yield total [C1]lactate. The value of *E* can be calculated from Table 4 using eqn. (19) for *r*₆.

$$r_6 = \frac{\{(m/z\ 222) + (m/z\ 221) + (m/z\ 220) + (m/z\ 219) - [C6]\text{lactate}\}}{[C6]\text{lactate}} \quad (19)$$

RESULTS

Experiment 1A: standard PPP measurements with [1-¹³C]glucose and [6-¹³C]glucose

Parallel incubations with [1-¹³C]glucose and [6-¹³C]glucose can be utilized to measure PPP activity in cultured cells [6]. The PPP activity measured in cultured glioma cells was stimulated from a basal activity of *P* = 7.1 ± 2.5 % to *P* = 37.2 ± 2.9 % by exposure to 5 μM PMS (Table 1). These values were considered the standard for validation of other PPP measurements using [6-¹³C,1,6,6-²H₂]glucose, [1,5,6-¹³C₃]glucose and [1,6-¹³C₂,6,6-²H₂]glucose.

Experiment 1B: studies with [6-¹³C,1,6,6-²H₂]glucose

In an attempt to measure PPP activity in a single incubation, we sought a multiply labelled glucose molecule that would produce two distinguishable lactate isotopomers when metabolized via

the PPP and glycolysis, one from carbon atom C-1 and the other from carbon atom C-6. With $[6\text{-}^{13}\text{C}, 1,6,6\text{-}^2\text{H}_2]\text{glucose}$, assuming no loss of ^2H atoms unless the adjacent carbon atom is also lost, glycolysis yields $[3\text{-}^{13}\text{C}, 3,3\text{-}^2\text{H}_2]\text{lactate}$ and $[3\text{-}^2\text{H}]\text{lactate}$ whereas metabolism through the PPP yields only $[3\text{-}^{13}\text{C}, 3,3\text{-}^2\text{H}_2]\text{lactate}$ (Table 5). These two lactate products can be distinguished by either g.c./m.s. or ^2H -n.m.r. spectroscopy. The calculated PPP activity with $[6\text{-}^{13}\text{C}, 1,6,6\text{-}^2\text{H}_2]\text{glucose}$ ($P = 28.9 \pm 2.2\%$; Table 1), was not consistent with the PPP activity measured in Experiment 1A ($P = 7.1 \pm 2.5\%$).

One possibility for this discrepancy is the selective loss of ^2H from C-1 of glucose as it is metabolized to lactate. Such an exchange of ^2H atoms from C-1 of glucose with ^1H atoms in water can be catalysed by phosphomannose isomerase, which catalyses the reversible formation of mannose 6-phosphate from fructose 6-phosphate [16,17]. The ^2H atoms from C-1 or C-6 of glucose can also exchange with ^1H atoms in water further down the glycolytic pathway at the pyruvate kinase step [15], and may exchange slowly from pyruvate via keto-enol tautomerization. The extent of exchange by these two mechanisms was tested by incubations with $[6\text{-}^{13}\text{C}, 6,6\text{-}^2\text{H}_2]\text{glucose}$, which should lose ^2H atoms only at the triose level and not via phosphomannose isomerase, and with $[1\text{-}^{13}\text{C}, 1\text{-}^2\text{H}]\text{glucose}$, which can lose ^2H via phosphomannose isomerase and at the triose level. In several different experiments with 9L glioma cells, values of $1 - \kappa_2 = 0.06\text{--}0.13$ and $1 - \mu = 0.14\text{--}0.38$ were observed, where $1 - \kappa_2$ is the fraction of ^2H atoms lost from each position at C-6 of glucose, presumably at the triose level, and $1 - \mu$ is the additional fraction of ^2H lost from C-1 of glucose, presumably as a result of phosphomannose isomerase activity. Therefore because $[6\text{-}^{13}\text{C}, 1,6,6\text{-}^2\text{H}_2]\text{glucose}$ could not be used to quantify PPP activity in a single incubation, studies using this isotopically substituted molecule were not pursued further.

Experiment 1C: studies with $[1,5,6\text{-}^{13}\text{C}_3]\text{glucose}$

In order to circumvent the complication caused by the loss of ^2H atoms, an attempt was made to develop a multiply labelled glucose molecule that did not contain ^2H atoms but that could still produce two distinguishable lactate isotopomers when metabolized via the PPP and glycolysis. Metabolism of $[1,5,6\text{-}^{13}\text{C}_3]\text{glucose}$ via glycolysis would yield equal amounts of $[3\text{-}^{13}\text{C}]\text{lactate}$ and $[2,3\text{-}^{13}\text{C}_2]\text{lactate}$; metabolism via the PPP would yield only $[2,3\text{-}^{13}\text{C}_2]\text{lactate}$ and some unlabelled lactate (Table 5). Thus $[2,3\text{-}^{13}\text{C}_2]\text{lactate}$ represents total glucose metabolized through glycolysis and PPP, whereas $[3\text{-}^{13}\text{C}]\text{lactate}$ represents glucose metabolized via glycolysis. The difference is due to metabolism via the PPP.

In glioma cells incubated with $[1,5,6\text{-}^{13}\text{C}_3]\text{glucose}$, there was surprisingly more $[1^3\text{C}]\text{lactate}$ than $[1^3\text{C}_2]\text{lactate}$, and the calculated PPP activity ($P = -8.9 \pm 2.6\%$) was significantly less than that ($P = 7.1 \pm 2.5\%$) measured in Experiment 1A. In a separate study, measured PPP activity increased from $P = -12.3 \pm 0.3\%$ to $P = 16.9 \pm 1.2\%$ when $5\text{ }\mu\text{M}$ PMS was added. Thus, although measurements with $[1,5,6\text{-}^{13}\text{C}_3]\text{glucose}$ did not provide an accurate measurement of PPP activity, they did detect changes in PPP activity.

To investigate whether selective loss of the $5\text{-}^{13}\text{C}$ label could account for our discordant results with $[1,5,6\text{-}^{13}\text{C}_3]\text{glucose}$, we incubated glioma cells with $[1\text{-}^{13}\text{C}]\text{glucose}$, $[5\text{-}^{13}\text{C}]\text{glucose}$ and $[6\text{-}^{13}\text{C}]\text{glucose}$ in three parallel incubations. PPP activity was calculated with $[1\text{-}^{13}\text{C}]\text{glucose}$ and either $[5\text{-}^{13}\text{C}]\text{glucose}$ ($P = 9.4 \pm 2.3\%$) or $[6\text{-}^{13}\text{C}]\text{glucose}$ ($P = 7.1 \pm 2.5\%$) (Table 1). Clearly there was no selective loss of ^{13}C from C-5 of glucose, thus ruling

out this explanation for the unexpected results with $[1,5,6\text{-}^{13}\text{C}_3]\text{glucose}$. Therefore because $[1,5,6\text{-}^{13}\text{C}_3]\text{glucose}$ could not be used to quantify PPP activity, studies using this molecule were not pursued further.

Experiment 1D: studies with $[1,6\text{-}^{13}\text{C}_2, 6,6\text{-}^2\text{H}_2]\text{glucose}$

We next sought a glucose molecule labelled in only the C-1 and C-6 positions that would not be affected by phosphomannose isomerase-catalysed exchange of ^2H atoms. $[1,6\text{-}^{13}\text{C}_2, 6,6\text{-}^2\text{H}_2]\text{Glucose}$ differs from $[6\text{-}^{13}\text{C}, 1,6,6\text{-}^2\text{H}_2]\text{glucose}$ only in having $^{13}\text{C}^1\text{H}$ instead of $^{12}\text{C}^2\text{H}$ at position 1 of glucose, thus avoiding the large and variable loss of the ^2H atom from C-1 of glucose. Measurements with $[1,6\text{-}^{13}\text{C}_2, 6,6\text{-}^2\text{H}_2]\text{glucose}$ yielded $P = 9.4 \pm 2.1\%$, which was similar to the parallel incubations with $[1\text{-}^{13}\text{C}]\text{glucose}$ and $[6\text{-}^{13}\text{C}]\text{glucose}$ (Table 1). This activity was unchanged [$P = 9.4 \pm 2.0\%$] when the glucose concentration was halved, from 5.5 mM to 2.8 mM , and PPP activity increased to $P = 30.6 \pm 1.9\%$ when $5\text{ }\mu\text{M}$ PMS was added to the incubation medium.

Measurements of PPP activity with $[1,6\text{-}^{13}\text{C}_2, 6,6\text{-}^2\text{H}_2]\text{glucose}$ assume that ^2H loss from each position is independent of ^2H loss from the other position, so that the amount of $[6]\text{lactate}$ that has lost two ^2H atoms can be calculated from the amount of lactate that has lost zero or one ^2H atom [eqn. (5)]. (If this correction were not made, $[6]\text{lactate}$ that has lost both ^2H atoms would appear as $[1]\text{lactate}$, causing an underestimation of PPP activity.) Independent loss is expected because pyruvate kinase-catalysed ^2H loss is not stereospecific [15]. Non-random loss could occur, however, if both ^2H atoms were lost simultaneously by some process. This does not occur in any reaction of glycolysis or the PPP, but the assumption may not be strictly valid if significant amounts of pyruvate enter the Krebs citric acid cycle and then leave via phosphoenolpyruvate carboxykinase.

The randomness of ^2H loss was tested in parallel incubations with $[6\text{-}^{13}\text{C}, 6,6\text{-}^2\text{H}_2]\text{glucose}$, $[1\text{-}^{13}\text{C}, 1\text{-}^2\text{H}]\text{glucose}$ and $[1,6\text{-}^{13}\text{C}_2, 6,6\text{-}^2\text{H}_2]\text{glucose}$. With $[6\text{-}^{13}\text{C}, 6,6\text{-}^2\text{H}_2]\text{glucose}$, $[6]\text{lactate}$ that has lost both ^2H atoms can be observed directly, because the resulting $[1^3\text{C}]\text{lactate}$ does not show any interference from $[1]\text{lactate}$. In the absence of PMS, $[1^3\text{C}, ^2\text{H}_0]\text{lactate}$ was 0.9% of total labelled lactate and $P = 4.0 \pm 0.4\%$ with calculated data, and $[1^3\text{C}, ^2\text{H}_0]\text{lactate}$ was 3.2% of total labelled lactate and $P = 6.1 \pm 0.3\%$ with observed data. With $[1,6\text{-}^{13}\text{C}_2, 6,6\text{-}^2\text{H}_2]\text{glucose}$, $P = 2.5 \pm 0.7\%$. In the presence of PMS, $P = 33.7 \pm 0.7\%$ with calculated data and $37.7 \pm 0.6\%$ with observed data, compared with $P = 36.9 \pm 0.9\%$ with $[1,6\text{-}^{13}\text{C}_2, 6,6\text{-}^2\text{H}_2]\text{glucose}$. Thus, although non-random ^2H loss may introduce small errors in absolute quantification of PPP activity, changes in PPP activity can be measured accurately with $[1,6\text{-}^{13}\text{C}_2, 6,6\text{-}^2\text{H}_2]\text{glucose}$.

When the calculations in the preceding paragraph were performed with a secondary isotope effect of 15% , $R_{12} = 1.15$, $[1^3\text{C}, ^2\text{H}_0]\text{lactate}$ increased from 0.9 to 1.1% of total labelled lactate with calculated data. With $[1\text{-}^{13}\text{C}, 1\text{-}^2\text{H}]\text{glucose}$ and $[6\text{-}^{13}\text{C}, 6,6\text{-}^2\text{H}_2]\text{glucose}$, P increased from 4.0 ± 0.4 to $4.5 \pm 0.4\%$ in the absence of PMS and from 33.7 ± 0.7 to $34.3 \pm 0.7\%$ in the presence of PMS. With $[1,6\text{-}^{13}\text{C}_2, 6,6\text{-}^2\text{H}_2]\text{glucose}$, P increased from 2.5 ± 0.7 to $2.8 \pm 0.7\%$ in the absence of PMS, and from 36.9 ± 0.9 to $37.1 \pm 0.9\%$ in the presence of PMS. Isotope effects therefore have a negligible effect on the calculated PPP activity.

Experiment 2: Isotopic dilution study with $[1,6\text{-}^{13}\text{C}_2, 6,6\text{-}^2\text{H}_2]\text{glucose}$

To determine the effect of moderate isotope dilution on PPP measurements with $[1,6\text{-}^{13}\text{C}_2, 6,6\text{-}^2\text{H}_2]\text{glucose}$, cultured 9L cells were incubated with 5.5 mM $[1,6\text{-}^{13}\text{C}_2, 6,6\text{-}^2\text{H}_2]\text{glucose}$ (100%) or

Figure 2 Time course of PPP activity in cultured 9L glioma cells

The PPP activity, measured by the parameter P , was quantified by g.c./m.s. analysis of lactate produced from the metabolism of $[1,6-^{13}\text{C}_2,6,6-^2\text{H}_2]\text{glucose}$ in control cells (□) and in cells exposed to $5\ \mu\text{M}$ PMS between hours 2 and 3 (○). The first and second downward-pointing arrows indicate PMS addition and removal respectively.

with $4.4\ \text{mM}$ $[1,6-^{13}\text{C}_2,6,6-^2\text{H}_2]\text{glucose}$ (80%) plus $1.1\ \text{mM}$ unlabelled glucose (20%). Baseline PPP activities of $P = 6.4 \pm 0.3\%$ and $P = 5.8 \pm 0.7\%$ increased to $P = 43.5 \pm 1.8\%$ and $P = 41.8 \pm 3.6\%$ in the presence of $5\ \mu\text{M}$ PMS (Table 1). The presence of 20% unlabelled glucose therefore did not interfere with the measurement of PPP activity.

Experiment 3: time-course study with $[1,6-^{13}\text{C}_2,6,6-^2\text{H}_2]\text{glucose}$

In order to monitor the time course of PPP stimulation by PMS, incubation buffer containing $5.5\ \text{mM}$ $[1,6-^{13}\text{C}_2,6,6-^2\text{H}_2]\text{glucose}$ was replaced every hour for 5 h. In one set of four wells, PMS was added during the third 1 h incubation period to stimulate PPP activity. PPP activity increased in the presence of PMS from $P = 4.9 \pm 2.0$ to $34.3 \pm 2.4\%$ and then gradually declined to baseline levels after PMS was removed, whereas in a parallel set of four control wells, the PPP activity remained at $P = 5.2\text{--}6.3\%$ (Figure 2). This demonstrates the usefulness of $[1,6-^{13}\text{C}_2,6,6-^2\text{H}_2]\text{glucose}$ in measuring PPP activity repetitively in a single sample of cultured cells.

DISCUSSION

The goal of this study was to quantify PPP activity in a single incubation with the sensitivity of g.c./m.s. analysis. PPP activities measured with each of the three novel isotopically substituted glucose molecules were compared with activities measured with $[1-^{13}\text{C}]\text{glucose}$ and $[6-^{13}\text{C}]\text{glucose}$ [6].

The high PPP activity calculated with $[6-^{13}\text{C},1,6,6-^2\text{H}_3]\text{glucose}$ can be explained by ^2H loss from the C-1 position of glucose. Although such an exchange can be catalysed by phosphomannose isomerase, our data are not sufficient to prove its involvement or to exclude other possible mechanisms.

The unexpected results with $[1,5,6-^{13}\text{C}_3]\text{glucose}$ are difficult to explain. Calculations based on the assumption that the C-5 and C-6 atoms of glucose are not lost during its metabolism to lactate yielded a negative PPP activity. The similarity between the results with $[5-^{13}\text{C}]\text{glucose}$ and $[6-^{13}\text{C}]\text{glucose}$ (Table 1) rules out

the possibility of selective loss of either C-5 or C-6 of glucose, or of lactate C-2 or C-3. Another mathematical possibility is that C-2 and C-3 of lactate are lost to an equal extent, but in each case the other C atom remains in (or returns to) lactate. A 5% loss in each position would yield numbers similar to our results. It is difficult to conceive of a pathway, however, where such a loss would occur. The proposed L-type non-oxidative pathway does not provide a mechanism for separate loss of C-5 and C-6 [18].

In contrast with the results with $[6-^{13}\text{C},1,6,6-^2\text{H}_3]\text{glucose}$ and $[1,5,6-^{13}\text{C}_3]\text{glucose}$, $[1,6-^{13}\text{C}_2,6,6-^2\text{H}_2]\text{glucose}$ yielded accurate measurements of PPP activity. By labelling glucose in both the C-1 and C-6 positions with distinguishable labels, PPP activity was quantified in a single incubation by g.c./m.s. analysis of the lactate extruded from the cells. The PPP activity was accurately measured in the presence of 20% unlabelled glucose (Experiment 2), when PPP activity was stimulated by PMS (Experiment 1D) and repetitively in a single population of cells (Experiment 3). With $[1,6-^{13}\text{C}_2,6,6-^2\text{H}_2]\text{glucose}$ it is also feasible to quantify PPP activity from other metabolites such as pyruvate, ethanol or alanine.

Measurements of PPP activity with $[1,6-^{13}\text{C}_2,6,6-^2\text{H}_2]\text{glucose}$ are subject to two limitations. First, in the presence of a large amount of unlabelled lactate, the 'tail' produced by the isotopic composition of the Me_3Si derivative could interfere with accurate determination of labelled lactate. In this case, the *n*-propylamide heptafluorobutyrate derivative should be used in order to reduce the isotopic background [19]. Second, in contrast with methods where $^{14}\text{CO}_2$ production is used for PPP quantification, our method relies on measuring the loss of a label. Therefore, the fractional enrichment must be known precisely for accurate measurement. Even if the precise enrichment is not known, however, relative changes in PPP activity can still be measured accurately.

One advantage of this method is its possible applicability to the measurement of PPP activity *in vivo*. For example, we have recently shown that $[1,6-^{13}\text{C}_2,6,6-^2\text{H}_2]\text{glucose}$, when combined with microdialysis techniques [20], allows *in vivo* regional PPP activity in the brain of conscious rats to be monitored [7]. Measurements revealed a basal PPP activity of $P = 3.5 \pm 0.4\%$ ($n = 4$) in rat forebrain, which could be stimulated by addition of PMS to the perfusion stream. This unique methodology should help to improve our understanding of the operation and regulation of this pathway in intact tissue, such as the brain, under both normal and neuropathological conditions.

Accuracy of absolute versus relative PPP activity

All data in this study were reported as means $\pm$ S.D., which gives an estimate of random errors in the data. In addition, any method based on isotopic enrichment may have systematic errors in the measurements. For PPP measurements with $[1,6-^{13}\text{C}_2,6,6-^2\text{H}_2]\text{glucose}$, errors in ^2H enrichment will cause errors mainly in the calculated values of κ_1 and κ_2 , so the accuracy of PPP activity is primarily affected by ^{13}C enrichment, which should be known within a few tenths per cent. Uncertainty in fractional enrichment will not affect the measured differences in PPP activity when altered metabolic conditions or various cell lines are compared.

Another source of systematic error is the possible loss of both ^2H atoms in excess of that calculated with the assumption of independent loss of each ^2H atom. Although a slight excess of ^2H loss was observed in 9L cells, stimulation of PPP activity by PMS was measured accurately with $[1,6-^{13}\text{C}_2,6,6-^2\text{H}_2]\text{glucose}$. Owing to the possibility of non-random ^2H loss, there may be a small uncertainty in the absolute PPP activity, possibly a few percent, in addition to uncertainty in the ^{13}C fractional enrichment.

Possible problems due to tissue heterogeneity

Larrabee [11] has pointed out problems that may occur in heterogeneous tissues, where two cell types differ in the percentage of glucose metabolized via the PPP and that metabolized to CO_2 . The proposed solution to this problem involves following the time course of $^{14}\text{CO}_2$ production from $[1\text{-}^{14}\text{C}]\text{glucose}$ and $[6\text{-}^{14}\text{C}]\text{glucose}$; the lower limit for PPP activity is $([\text{C}1]\text{CO}_2 - [\text{C}6]\text{CO}_2)_{\text{max.}}$, and the upper limit is steady-state $[\text{C}1]\text{CO}_2$ [21]. The formulation of Larrabee, which assumes that PPP activity remains constant over time, is valid for systems in steady state, but is not valid when PPP activity changes over time because of either unspecified causes [9] or deliberate stimulation, for example with PMS (Figure 2). In contrast, $[1,6\text{-}^{13}\text{C}_2,6,6\text{-}^2\text{H}_2]\text{glucose}$ has been used to measure dynamic PPP activity (Experiment 3, Figure 2) with a time resolution of 1 h, and shorter times are easily accommodated. In cultured cells where heterogeneity is not a problem, the method presented here is preferable.

Loss of ^2H atoms from glucose

The extensive loss of ^2H label from C-1 of glucose prevents the use of $[6\text{-}^{13}\text{C},1,6,6\text{-}^2\text{H}_3]\text{glucose}$ for quantifying PPP activity, and it raises questions about interpreting the loss of ^3H from $[1\text{-}^3\text{H}]\text{glucose}$ in erythrocytes as a measurement of PPP activity [22].

The values of κ_2 and μ are consistent with observations in yeast, where the ^2H content of ethanol produced from $[6,6\text{-}^2\text{H}_2]\text{glucose}$ was analysed [16]. Analysis of these data for metabolism of $[6,6\text{-}^2\text{H}_2]\text{glucose}$ in H_2O yields $1 - \kappa_2 \approx 0.10$, and for unlabelled glucose metabolism in $^2\text{H}_2\text{O}$ $1 - \kappa_2 = 0.27$ and $1 - \mu = 0.57 - 0.62$.

Comparison with C-2-labelled glucose methods

Another approach to the measurement of PPP activity in a single incubation is to use $[2\text{-}^{13}\text{C}]\text{glucose}$ or $[2\text{-}^{14}\text{C}]\text{glucose}$, and to determine the distribution of ^{13}C or ^{14}C in lactate [2]. For $[2\text{-}^{14}\text{C}]\text{glucose}$ this entails degrading lactate and determining the amount of radioactivity in each carbon atom of the molecule, a very time-consuming procedure. For $[2\text{-}^{13}\text{C}]\text{glucose}$ the distribution of ^{13}C isotope can be determined by ^{13}C -n.m.r. spectroscopy. This method, however, is much less sensitive than g.c./m.s., thus requiring more isotopically substituted glucose and more time to analyse each sample.

In addition to these methodological considerations, the use of $[2\text{-}^{13}\text{C}]\text{glucose}$ is based on two assumptions which are not strictly correct, and the amount of deviation from ideality is uncertain. First, pentose phosphates are assumed to be converted into glycolytic intermediates by the most efficient combination of reactions catalysed by transaldolase and transketolase. The effects of other rearrangements of the pentose carbon atoms on the distribution of ^{13}C in lactate have not been calculated. In fact, the formation of $[2\text{-}^{14}\text{C}]\text{ribose}$ from $[2\text{-}^{14}\text{C}]\text{glucose}$ in muscle tissues [23] and in bacteria [24] and of $[1\text{-}^{14}\text{C}]\text{deoxyribose}$ from $[1\text{-}^{14}\text{C}]\text{glucose}$ in bacteria [24] suggests that the non-oxidative branch is readily reversible.

Second, calculations of PPP activity from ^{13}C distribution in lactate assume complete recycling of PPP products into the PPP. Incomplete recycling affects not only the distribution of ^{13}C in lactate, but also the relationship between the parameters that we

have called P and T . The error introduced by this assumption can be calculated for given values of P and Y by utilizing the approach of Wood and Katz [25].

In summary, the use of $[1,6\text{-}^{13}\text{C}_2,6,6\text{-}^2\text{H}_2]\text{glucose}$ provides a novel approach for measuring PPP activity and allows this activity to be followed over time. The value of P obtained with $[1,6\text{-}^{13}\text{C}_2,6,6\text{-}^2\text{H}_2]\text{glucose}$ represents the relative rate of PPP activity compared with glycolysis. In order to measure net PPP flux, it is necessary to consider recycling (T versus P in Table 4) and to correct for the amount of glucose consumed or lactate produced. Although cultured tumour cells were used to validate and evaluate this molecule for monitoring the PPP, this method could be used to investigate this pathway in a multitude of cell types and under a wide variety of conditions. When combined with microdialysis techniques [20], preliminary observation indicates its possible applicability *in vivo* in rat brain for measurements of PPP activity [7].

We thank Dr. Anthony Serianni of Omicron Biochemicals for help in synthesizing the glucose isotopes used in this study and Dr. Thomas Krick for valuable advice regarding g.c./m.s. techniques. Rat 9L glioma cells were provided by The Brain Tumor Research Center, University of California, San Francisco, CA, U.S.A. These studies were supported in part by a Research Investigation Grant from the American Cancer Society (grant no. BE-149), an Institutional Research Grant (no. IN-40-32) to the University of Michigan Cancer Center from the American Cancer Society, NIH Grants P20 NS31114, R29 CA59009 and R29 HL42168, the American Lebanese Syrian Associated Charities (ALSAC), the Cancer Center Support (CORE) grant P30CA21765 and the John and Suzanne Munn Endowed Research Fund.

REFERENCES

- Katz, J. and Wood, H. G. (1960) *J. Biol. Chem.* **235**, 2165–2177
- Wood, H. G., Katz, J. and Landau, B. R. (1963) *Biochem. Z.* **338**, 809–847
- Katz, J., Landau, B. R. and Bartsch, G. E. (1966) *J. Biol. Chem.* **241**, 727–740
- Hothersall, J. S., Baquer, N. Z., Greenbaum, A. L. and McLean, P. (1979) *Arch. Biochem. Biophys.* **198**, 478–492
- Jolley, R. L., Cheldelin, V. H. and Newbergh, R. W. (1958) *J. Biol. Chem.* **233**, 1289–1294
- Kingsley-Hickman, P. B., Ross, B. D. and Krick, T. (1990) *Anal. Biochem.* **185**, 235–237
- Ross, B. D., Ben-Yoseph, O., Camp, D. M. and Robinson, T. E. (1993) *Soc. Neurosci. Abstr.* **19**, Part 2, 1268
- Wu, J., Serianni, A. S. and Bondo, P. B. (1992) *Carbohydr. Res.* **226**, 261–269
- Mitchell, S. L., Ross, B. D., Krick, T. and Garwood, M. (1989) *Biochem. Biophys. Res. Commun.* **158**, 474–479
- Lide, D. R. (ed.) (1991) *CRC Handbook of Chemistry and Physics*, 72nd edn., CRC Press, Boca Raton, FL
- Larrabee, M. G. (1990) *Biochem. J.* **272**, 127–132
- Landau, B. R., Bartsch, G. E., Katz, J. and Wood, H. G. (1964) *J. Biol. Chem.* **239**, 686–696
- Landau, B. R. and Katz, J. (1964) *J. Biol. Chem.* **239**, 697–704
- Landau, B. R. and Bartsch, G. E. (1966) *J. Biol. Chem.* **241**, 741–749
- Robinson, J. L. and Rose, I. A. (1972) *J. Biol. Chem.* **247**, 1096–1105
- Saur, W. K., Peterson, D. T., Halevi, E. A., Crespi, H. L. and Katz, J. J. (1968) *Biochemistry* **7**, 3537–3546
- Saur, W. K., Crespi, H. L., Halevi, E. A. and Katz, J. J. (1968) *Biochemistry* **7**, 3529–3536
- Longenecker, J. P. and Williams, J. F. (1980) *Biochem. J.* **188**, 847–857
- Tserng, K.-Y., Gillilan, C. A. and Kalhan, S. C. (1984) *Anal. Chem.* **56**, 517–523
- Ungerstedt, U. (1991) in *Microdialysis in the Neurosciences: Techniques in the Behavioral and Neural Sciences* (Robinson, T. E. and Justice, J. B., eds.), vol. 7, pp. 3–22, Elsevier Science Publishing Co., New York
- Larrabee, M. G. (1989) *J. Biol. Chem.* **264**, 15875–15879
- Newmark, R. D., Un, S., Williams, P. G., Carson, P. J., Morimoto, H. and Klein, M. P. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 583–587
- Green, M. R. and Landau, B. R. (1965) *Arch. Biochem. Biophys.* **111**, 569–575
- Katz, J. and Rognstad, R. (1967) *Biochemistry* **6**, 2227–2247
- Wood, H. G. and Katz, J. (1958) *J. Biol. Chem.* **233**, 1279–1282