

In vivo Quantitative Glucose Metabolite Analysis in Yeast Using ¹³C Labeled Sugars to Determine Enzyme Channeling

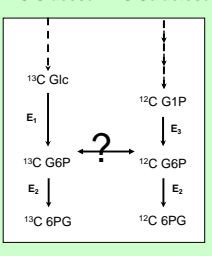
Michael A. Grayson[†], James J. Walters[†], Maureen M. Hughes[‡], Michael L. Gross[†], James Bashkin^{*}, Daniel H. Kohl[‡], Washington University, St Louis, MO 63130
[†]Department of Chemistry [‡]Department of Biology ^{*} Consultant, St Louis MO

Overview:

To determine whether the enzymes in a pathway channel metabolic intermediates, quantitative determination of specific metabolites *in vivo* is required. The enzyme system examined is the oxidative limb of the pentose phosphate pathway in a mutant yeast, *Saccharomyces cerevisiae*, in which galactose metabolism is not repressed by glucose. Thus, by feeding this yeast strain varying ratios of ¹³C labeled glucose and ¹³C galactose and measuring the related steady-state ratios of glucose-6-phosphate (¹³C-¹²C G6P) and 6-phosphogluconate (¹³C-¹²C 6PG) in the cell, we can deduce the extent of channeling of pathway intermediates.

IA-Yeast

¹³C Glucose ¹²C Galactose



Methods:

• Yeast Incubation

Yeast strain YM6880 was incubated for 30 minutes with and without 4mM Sodium m-ketone to inhibit decarboxylation of pyruvate and 2mM Phenazine methosulfate to reoxidize reduced NADPH. The yeast was also incubated with varying millimolar quantities of ¹³C glucose or ¹³C glucose and ¹³C galactose. Sugar monophosphates from the incubations were extracted by standard methods using a hot buffered ethanol procedure to remove proteins.

Methods:

• Liquid Chromatography

Samples from yeast extracts were separated using strong anion-exchange liquid chromatography on a Dionex Carbopac PA1 column with solvent A: 50 mM sodium hydroxide (NaOH) and solvent B: a mixture of 50 mM NaOH and 600 mM sodium acetate, at a flow rate of 1 mL/min. Initial conditions were 85% A and 15% B with a linear gradient to 65% B over 40 min. The chromatographic effluent was desalted with a Dionex CMD desalter. Due to low analyte concentration, direct LC/MS analysis via a split flow to the mass spectrometer was not feasible. Consequently, sample fractions were collected and vacuum dried for later analysis.

Methods:

• Mass Spectrometry

The dried LC fractions were reconstituted in HPLC grade methanol containing 0.5% acetic acid by volume. The same solution was used to infuse the samples with the syringe pump on the Micromass (now Waters) Q-ToF mass spectrometer. Sample solutions had a pH in the range of 2.5 to 3.5. Three aliquots of 4μL of the reconstituted LC fractions were injected using a modified 'direct injection' inlet. The mass spectrometer was operated in negative ion mode at a resolving power of 10,000. Under these conditions, the masses of the sugar monophosphates were resolved from the chemical noise in the reconstituted samples. However, MS/MS data were recorded to confirm that the sample MS/MS spectra matched those of authentic standards.

Methods:

• Data Analysis

Reconstructed ion current profiles were created for the ¹²C and ¹³C masses of G6P, 259 and 265 Da respectively, and the ¹²C and ¹³C masses of 6PG, 275 and 281 Da respectively. Areas under the profiles were integrated using data processing tools available in the Micromass (now Waters) software data analysis suite.

Discussion of Experimental Issues:

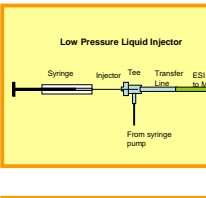
• Desalting LC Eluent

The Dionex CMD desalter operates optimally below 350 mM salt concentration. Elution of the 6PG sugar monophosphate requires gradients with salt concentrations very close to this range. Desalting with a regenerant solution of aqueous trifluoroacetic acid resulted in the formation of sodium trifluoroacetate clusters in the ESI spectrum of the reconstituted LC fractions. This chemical noise completely suppressed ionization of the low level concentration of the sugar monophosphates. Subsequently, a 50mM solution of aqueous sulfuric acid was used for the desalter regenerant. This resulted in some sulfuric acid and sulfate salt peaks in the ESI spectrum, but ionization of the analytes was sufficient to obtain useful data.

Discussion of Experimental Issues:

• Direct Injection to ESI Source

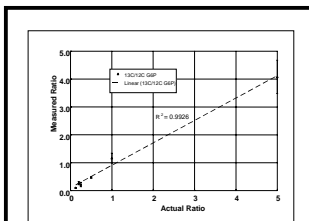
Initial analyses were conducted using the switching valve and injection loop supplied with the instrument. However, the sugar monophosphates are 'sticky' and a variety of artifacts occurred using this sample introduction method. Subsequently, a simple tee injection inlet was constructed which removed all PEEK transfer lines and valves from the sample flow and decreased the distance from the syringe needle to the ESI spray to as short as possible.



Discussion of Experimental Issues:

• Mass Spectrometry

A variety of spray solvents were examined to optimize the ionization of the sugar monophosphates. Although, at first it appears counter-intuitive, the weakly acidic methanol solution provided the most intense M-H ions. In addition, it minimized the appearance of isolated adducts in the mass spectrum, forcing more ionization into the M-H ion. Finally, the collision energy in the cell was reduced to about half of its normal value to further enhance the molecular ion, since the sugar monophosphates fragment very easily.



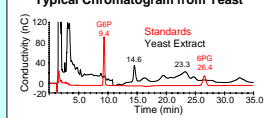
Authentic standards of ¹³C and ¹²C G6P were mixed in various ratios and chromatographed, desalted, collected, dried and submitted to MS analysis in the exact procedure used to analyze yeast extracts. Results of several trials are shown here.

Discussion of Results:

• Chromatographic Data

Typical chromatographic data from the mutant yeast indicate that the sugar monophosphates appear in a complex mixture of compounds. The large peak at ~15 minute elution does not correspond to a compound of expected interest, but was collected and analyzed along with the fractions containing G6P and 6PG.

Typical Chromatogram from Yeast



The retention times of G6P and 6PG were determined by injection of a mixture of authentic standards of the two sugar monophosphates. The unknown peak at 14.6 minutes elution was identified as a seven carbon sugar monophosphate.

Incubation Protocols:

Five separate incubations of the yeast were performed with the following concentrations of glucose and galactose

- I - 0 mM ¹³C Glucose, 0 mM ¹²C Galactose
- II - 2 mM ¹³C Glucose, 0 mM ¹²C Galactose
- III - 2 mM ¹³C Glucose, 2 mM ¹²C Galactose
- IV - 8 mM ¹³C Glucose, 0 mM ¹²C Galactose
- V - 8 mM ¹³C Glucose, 2 mM ¹²C Galactose

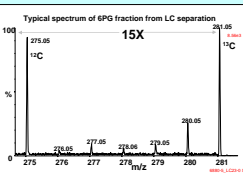
Discussion of Results:

• Mass Spectral Data

Initial experiments detected only trace levels of G6P in any of the various incubation experiments.

Easily detectable levels of 6PG were found in the incubation without either sugar.

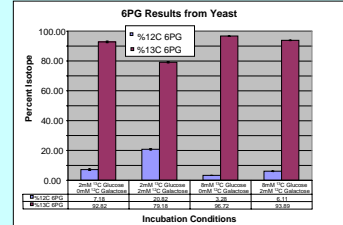
All of the incubations that contained either glucose or galactose had significant quantities of 6PG present.



The pattern of peaks between the ¹²C and ¹³C ions indicates that some molecules are formed with varying numbers of ¹³C atoms during incubation of this mutant yeast.

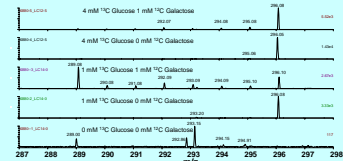
Summary:

- A method for the determination of the ratio of sugar monophosphates from yeast extracts has been developed
- Initial analyses of yeast extracts indicate that
 - > G6P is present in trace amounts
 - > The ¹³C content of the mixture of sugars used for incubation is reflected in the 6PG sugar monophosphate
 - > A seven carbon sugar monophosphate was discovered in the yeast products
- Additional experiments are underway to conclusively determine if channeling does occur in the mutant yeast, *Saccharomyces cerevisiae*
- The method will be used in the study of channeling in other biological systems



While the relative amounts of 6PG detected did not reflect exactly the relative amounts of sugars in the various incubations, they did reflect the trends of sugar concentration.

7 Carbon Sugar Monophosphate Isotope Distribution from Yeast Incubated with Different Amounts of Sugars (¹²C ion at 289 Da)



As in the case of the 6PG, the pattern of peaks between the ¹²C and ¹³C ions of the seven carbon sugar monophosphate indicates that some molecules are formed with varying numbers of ¹³C atoms during incubation of this mutant yeast.

The seven carbon monophosphate is not present in the yeast incubated without G6P and 6PG.