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Laboratory exercise

The pentose phosphate pathway in the yeasts *Saccharomyces cerevisiae* and *Kloeckera apiculata*, an exercise in comparative metabolism for food and wine science students

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Abstract

Comparative cellular metabolism can be a difficult area of biochemistry to teach in the undergraduate laboratory class. Student practicals involving animal tissues generally require approval from animal ethic committees, and the relevance for students whose primary interest in biochemistry is in the area of food and wine sciences, is often questioned. In this report, we present an undergraduate practical exercise in which glucose catabolism via the pentose phosphate pathway is compared in two types of yeast with direct relevance to the wine and food industries, *Saccharomyces cerevisiae* and *Kloeckera apiculata*. The exercise is carried out as a demonstration to second year undergraduate students, studying metabolic biochemistry. It is of some value in that it illustrates comparative cellular metabolism in wine yeasts and introduces the students to the safe use of radioisotopes.

Keywords: Pentose phosphate pathway; Wine yeasts; *Saccharomyces cerevisiae*; *Kloeckera apiculata*; *Hanseniaspora uvarum*; [1-^{14}C]-glucose; Carbohydrate metabolism

1. Introduction

The pentose phosphate pathway, also called the hexose monophosphate and the phosphogluconate pathway serves two functions in the cell. It provides reducing equivalents in the form of NADPH for lipid and other biosynthetic purposes, and it provides pentoses for the synthesis of nucleic acids, glycoproteins and other substances [1]. Unlike glycolysis which proceeds by formation of fructose-1, 6-bisphosphate, glucose-6-phosphate is not further phosphorylated. After conversion to phosphogluconate, carbon 1 of glucose is lost in an oxidative decarboxylation reaction to produce ribulose-5-phosphate which in turn produces ribose-5-phosphate for nucleic acid synthesis (Fig. 1). The loss of carbon dioxide from carbon 1 of glucose may be taken as an approximate measure of the amount of glucose metabolised via the pentose phosphate pathway. Carbon dioxide arising from alcoholic fermentation of glucose originates from carbons 3 and 4. Carbon dioxide produced as a result of respiration can arise from all of the carbon atoms of the glucose, including carbon 1, but can be inhibited by potassium cyanide.

Using [1-^{14}C]-glucose, the amount of glucose catabolised via the pentose phosphate pathway can be estimated from the amount of [^{14}C]-carbon dioxide released. Given that the stoichiometry of the equation for the oxidative portion of the pentose phosphate pathway is such that one molecule of CO_2 is produced per molecule of glucose catabolised, (1), then the specific activity of the released CO_2 will be the same as that of glucose.

$$\text{Glucose} + 2\text{NADP}^+ \rightarrow \text{CO}_2 + 2\text{NADP} + \text{H}^+ + \text{Ribose}. \quad (1)$$

Eq. (1), also shows that the specific activity of the CO_2 released is the ratio of μCi of released CO_2 to μmol of glucose consumed via the pentose phosphate pathway. The latter can now be calculated from the known specific activity of the added glucose.

We have developed an undergraduate practical exercise, conducted as a demonstration to illustrate the pentose phosphate pathway in two yeasts, *Saccharo-*

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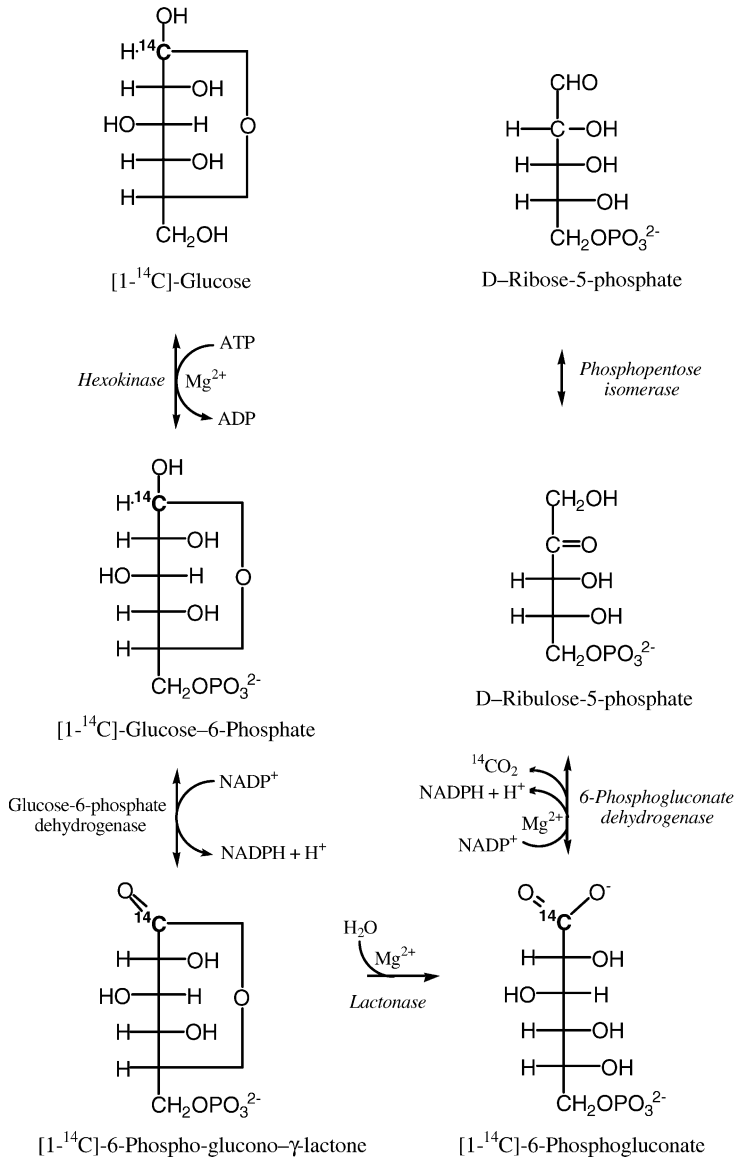

Fig. 1. The oxidative portion of the pentose phosphate pathway showing the origin of released carbon dioxide.

myces cerevisiae, normally used in brewing and baking, and *Kloeckera apiculata* (anamorph *Hanseniaspora uvarum*) a wild yeast commonly associated with the normal microflora of mature grapevine berries [2]. As the two yeasts differ considerably in ribonucleic acid content [3], they exhibit major differences for ribose synthesis and consequently show different requirements for glucose catabolism via the pentose phosphate pathway.

2. Materials and methods

Cultures of S. *cerevisiae* and *K. apiculata* may be obtained from various yeast culture collections around the world. Both organisms can also be readily isolated from either grape berries or wine making equipment [2]. We maintained the yeast culture on agar slopes of Malt Yeast Extract Agar (Difco, Australia), and when required sub-cultured the yeast into flasks of malt yeast extract broth (Difco, Australia) 12–18 h prior to the commencement of the exercise.

Broth cultures in the logarithmic phase of growth (determined by optical density estimations), are harvested using centrifugation (3000*g*, 20 min), and the wet weight recorded. The cells are resuspended in potassium phosphate buffer (10 mM, pH 6.3), to give a final yeast suspension of 5% (w/v).

[1-^{14}C]-Glucose was obtained from Amersham, Australia and the scintillation counting fluid used was Ultima-Gold (Canberra-Packard, Australia). Hyamine was also obtained from Canberra-Packard, Australia

while all other reagents were obtained from Sigma Aldrich Pty Ltd (Castle Hill, Australia). Tables 1 and 2 list equipment, consumable items and preparation of reagents required to conduct the exercise.

2.1. Experimental procedure

The reaction is carried out in a 10 cm headspace vial with a crimp seal and teflon linear (Alltech, Baulkham Hills, Australia). All manipulations of the radioactive material are done in a fume cupboard. The incubation mixture consists of 0.1 ml of the appropriate yeast culture and 10 µl of KCN (0.1 M) to inhibit respiration. The reaction vessel contains three glass beads to support a Durham tube containing the hyamine solution (0.1 ml, 1.0 M in methanol). [1-^{14}C]-Glucose (2.5 µCi/ml, 0.1 ml, 2.5 mM) is introduced into the incubation mixture via a syringe and the vial sealed to start the experiment. A second set of vials containing unlabelled glucose is also prepared at the same time. Both control and radiolabelled tubes are incubated for 30 min with gentle agitation at 30°C in a water bath.

The tubes containing the unlabelled glucose are used for the estimation of glucose catabolised using a commercially available glucose oxidase assay kit, prepared according to the manufacturers directions (Boehringer-Mannheim, Australia). An aliquot (0.1 ml) of the incubation mixture is removed to a centrifuge tube containing perchloric acid (0.4 M, 1.0 ml) and the contents mixed. The material is centrifuged (3000*g*, 5 min) and 0.2 ml of the supernatant removed. The acid-treated supernatant is then neutralised with disodium hydrogen phosphate (0.4 M, 0.2 ml), mixed, and then 2.5 ml of the glucose oxidase reagent added. The absorbance at 510 nm is read after 30 min and the glucose concentration determined with reference to a standard curve. The amount of glucose catabolised is assayed by measuring glucose at both the end and the start of the incubation period, using duplicate tubes containing unlabelled glucose. Additionally, samples of the yeast suspensions are assayed at the start of the incubation period to confirm that the amount of glucose present in the washed culture is negligible prior to the addition of the glucose. Any glucose detected is taken into account in the final calculation.

At the end of the incubation period, the carbon dioxide in tubes containing the [1-^{14}C]-glucose is driven-out of the incubation mixture by addition of sodium carbonate (0.1 M, 0.05 ml). This is then acidified with a volume of HCl (5 M, 0.05 ml) and the mixture incubated for a further 45 min at 30°C. The released CO_2 is absorbed by the strong organic base, hyamine, present in the Durham tube. This solution is then transferred to 5 ml of scintillant and the radioactivity assayed using liquid scintillation counting.

2.2. Calculation of glucose flux through the pentose phosphate pathway

Total glucose present at the start and end of the incubation period is calculated with reference to the standard curve. The total number of microcuries of $^{14}CO_2$ released for each sample is calculated using the counts per minute recorded and correction made for counting efficiency using an external standard. The students are requested to calculate the specific activity of the $^{14}CO_2$ released by relating the total microcuries of ^{14}C-glucose added to each sample to total µmol of glucose present at the start of incubation. As the specific activity of glucose and carbon dioxide are the same, the total µmol of $^{14}CO_2$ produced for each sample can be determined. From this, the percentage of the total

Table 1
Equipment and Consumable items required to set-up a demonstration to illustrate the pentose phosphate pathway in yeasts

Equipment
10 cm headspace vials (Alltech Part number 6655) with crimp seals (Alltech Part number 95756), containing three glass beads plus a Durham tube (one tube per incubation)
Shaking water bath set at 30°C (for incubations containing unlabelled glucose)
Shaking water bath set at 30°C in fume hood (for tubes containing radio labelled glucose)
Spectrophotometer set to 510 nm
Automatic pipettes
High speed centrifuge
Consumables
Glucose oxidase reagent (prepared according to manufacturers instructions)
Scintillation vials and lids
Centrifuge tubes
Pasteur pipettes and bulbs
Macrocuvettes (4.5 ml volume)
Contamination container for radioactive waste
Beaker containing disinfectant for disposal of waste yeast culture
1 ml syringes plus 23G needles
Disposable pipette tips (200 and 1000 µl)
Latex disposable gloves

Table 2
Details of reagents required to set-up a demonstration to illustrate the pentose phosphate pathway in yeasts

Yeast suspensions. Grow yeast for 12–18 h at 30°C in Malt broth with shaking until the $OD_{600\,nm}$ is approximately 0.2
Glucose (2.5 mM) (0.45 g glucose/1 l water)
Glucose (2.5 mM) containing 2.5 mCi of glucose-1-C^{14}/ml
Hyamine solution, 1 M in methanol
Perchloric acid (0.4 M) (23.6 ml Perchloric acid in 1 l water)
Potassium cyanide (0.1 M) (0.75 g KCN/100 ml water)
Scintillant, Packard Ultima Gold, in dispenser set at 5 ml
Sodium carbonate (0.1 M) (1.06 g Na_2CO_3/100 ml water)
Hydrochloric acid (5 M)
Disodium hydrogen phosphate (0.4 M) (28.34 g Na_2HPO_4/500 ml water)

glucose catabolised via the pentose phosphate pathway can be calculated.

3. Results and discussion

This exercise has been taught for over three years to second year undergraduate students enrolled in degree-level courses in wine & food sciences. The exercise was originally conducted with students working in pairs, but we found it more effective to teach the exercise as a demonstration. The demonstration commences with a pre-practical talk, during which time the use of radio-isotopes in metabolic studies and the theory of the pentose phosphate pathway is discussed. Some background information on non-*Saccharomyces* spoilage yeasts in wine production is also given. Prior to this exercise, students have completed two additional practical exercises on carbohydrate metabolism; the first of these investigates the suitability of different mono- and disaccharides for alcoholic fermentation while the second deals with the Pasteur effect. In combination with the lecture component, the students should therefore understand the function of the pentose phosphate pathway in the overall context of carbohydrate metabolism.

At the conclusion of the demonstration, students are provided with the raw data in the form of counts per minute of the hyamine solution, disintegrations per minute are then calculated with reference to a supplied counting efficiency curve. A sample calculation table that a student might prepare is illustrated in Table 3. In order to perform the calculations, students have to understand the stoichiometry of the equation for the oxidative portion of the pentose phosphate pathway. They also have to understand the origins of the carbon dioxide in the pentose phosphate pathway, respiration and alcoholic fermentation in yeast. Predicted results are that the culture of *K. apiculata* will catabolise less glucose via the pentose phosphate pathway than the culture of *S. cerevisiae*. In their practical reports, students have to comprehend why this is so, i.e. differences in the requirements for ribose synthesis.

Concepts which the students are expected to grasp at the conclusion of the exercise include:

- Stoichiometry of the oxidative portion of the pentose phosphate pathway,
- The pentose phosphate pathway as an alternative route to glycolysis for glucose catabolism,
- The two primary functions of the oxidative portion of the pentose phosphate pathway, i.e. formation of pentose sugars for nucleic acids and NADPH for lipid and other biosynthetic pathways,
- That yeast differ in their relative activity of the pentose phosphate pathway because of differing ribonucleic acid and/or lipid biosynthesis requirements,
- Use of radioisotope techniques in metabolic studies.

Typical study questions that might be used either before or after the practical session include the following:

- What function does the pentose phosphate pathway have in yeast cells?
- Why are there differences in activity of the pentose phosphate pathway in different types of yeast?
- Explain why the specific activities are the same for $^{14}CO_2$ and ^{14}C glucose?

Table 3
Sample calculation of glucose flux through the pentose phosphate pathway. Students are provided with the cpm values for the two cultures, the remainder of the table has to be calculated by the students

	Incubation	
	S. cerevisiae	*K. apiculata*
cpm[a]	30479	20731
Counting efficiency[b] (%)	95	95
dpm[c]	32083	21822
$^{14}CO_2$ released[d] (μCi)	0.01445	0.00983
$^{14}CO_2$ released[e] (μmol)	0.05781	0.03932
Glucose catabolised[f] (μmol)	0.25	0.25
Glucose catabolised via pentose phosphate pathway[g] (%)	23%	16%

[a] Counts per minute as recorded on scintillation counter.
[b] Percentage counting efficiency determined with reference to counting efficiency curve.
[c] Disintegrations per minute.
[d] Calculated by dividing the dpm value by 2.22×10^6.
[e] Calculated by dividing μCi $^{14}CO_2$ released by the specific activity, i.e. 0.25.
[f] The glucose catabolised is calculated by the students with reference to a standard curve that they have prepared.
[g] The amount of mmoles $^{14}CO_2$ released is divided by μmol glucose catabolised and multiplied by 100 in order to obtain this percentage.

- If 1-^{14}C glucose is catabolised in yeasts and muscle cells to ethanol and lactate, respectively, indicate the position of the radioactive label in the final products.

A source of variation in the results can be the growth stage yeast cultures. Both species should be in the logarithmic phase of growth, cultures that are in the stationary phase, have an overall slow rate of metabolism. If this is the case, little glucose is metabolised in the 30 min incubation period. However, this problem will not arise if the growth of the two cultures is monitored prior to the commencement of the demonstration. Normally, cultures inoculated with a starter culture 12–18 h prior to the start of the demonstration will be in the logarithmic phase of growth and suitable for the exercise.

As an alternative to using two different species of yeast, it may be possible to employ mutants that are deficient in one more of the enzymes of the pentose phosphate pathway, such as glucose-6-phosphate dehydrogenase deficient cells of *Saccharomyces cerevisiae* [4–6]. Alternatively, it could be possible to use mutant strains of *Escherichia coli*, [7]. Such mutant strains could of course be used as an adaptation of this exercise if it is not possible to obtain cultures of the wine yeasts. This however, is something that we have not attempted.

We have however, performed this experiment using human red blood cells which utilise NADPH to maintain haemoglobin in the reduced (Fe II) state. Errors are magnified with these cells as glucose consumption and CO_2 release is only 5–10% that of yeast under these conditions. Because of this, and also because of risk of infection, we do not recommend this procedure to be used with human blood. Furthermore, exercises involving animal tissues, generally require approval from various animal ethic committees [8], this of course is not the case if yeast cultures are used.

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