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Pathways of Glucose Metabolism in *Candida* 107, a Lipid-accumulating Yeast

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Phosphofructokinase was not detected in extracts of *Candida* 107 prepared in a variety of ways but was highly active in cells treated with toluene. Disruption of these cells destroyed activity of phosphofructokinase indicating that the enzyme is extremely labile. As patterns of labelling from [1-^{14}C]glucose and [6-^{14}C]glucose showed that 60% of glucose was metabolized via the pentose cycle, augmentation of this cycle is necessary to account for the high molar growth yields of this yeast. Phosphoketolases, reacting with xylulose 5-phosphate and fructose 6-phosphate, were found but the extent to which they contribute to glucose metabolism was not assessed.

INTRODUCTION

We have previously examined the intermediary metabolism of an oleaginous (fat-producing) yeast, *Candida* 107, and, because of the apparent absence of phosphofructokinase, concluded that glucose may be metabolized via the pentose cycle (Whitworth & Ratledge, 1975*a*). Metabolism of glucose by this cycle, although providing NADPH for fatty acid biosynthesis, would be inefficient with only 1 mol acetyl-CoA arising from 1 mol glucose. An alternative must exist to explain the high molar growth and lipid yields of this organism (Gill, Hall & Ratledge, 1977). The same conclusion was reached by Höfer and co-workers (Höfer, 1968; Höfer *et al.*, 1971) and by Brady & Chambliss (1967) working with another fat-accumulating yeast, *Rhodotorula gracilis* (now *Rhodosporidium toruloides*), in which phosphofructokinase also seemed to be absent. Höfer *et al.* (1969) suggested that phosphoketolase may be present to split xylulose 5-phosphate (formed by the transaldolase and transketolase reactions) into glyceraldehyde 3-phosphate and acetyl-CoA but did not look for this enzyme. However, Mazón, Gancedo & Gancedo (1974) found a phosphofructokinase in *Rhodotorula glutinis* which was active only under carefully controlled conditions, indicating that caution was needed in interpreting the previous results. When we repeated the latter workers' assay with extracts of *Candida* 107 we still failed to detect this enzyme and therefore began an examination of glucose metabolism in this yeast. Our results are presented here.

METHODS

Organisms and growth. Candida 107 Ruinen & Deinema 1964 and, as a control organism, *Saccharomyces cerevisiae* were grown at 30 °C in a medium consisting of (g l^{-1}): glucose, 12; KH_2PO_4, 7; Na_2HPO_4, 2; NH_4Cl, 1·7; $MgSO_4.7H_2O$, 1·5; yeast extract, 1·5; $CaCl_2.6H_2O$, 0·1; $FeCl_3.6H_2O$, 0·05; $ZnSO_4.7H_2O$, 0·0001; with a final pH of 5·5. The yeasts were usually grown in 100 ml medium in 250 ml conical flasks but occasionally in a chemostat as previously described (Gill *et al.*, 1977). Yeasts were harvested at the end of active growth by centrifuging at 10000 *g* for 5 to 10 min, washed once with 50 mM-phosphate buffer, pH 7·5, containing 2 mM-$MgCl_2$ and then resuspended in the same buffer solution to about 300 mg dry wt ml^{-1}.

Preparation of cell-free extracts. Conventional procedures were followed for disrupting cells (Hughes,

Wimpenny & Lloyd, 1971), the usual method being passage through a French press. Disrupted material was centrifuged at 45000 *g* for 30 min; solidified lipid was removed by filtering the supernatant solution through Whatman no. 1 filter paper.

Toluene lysis of yeast was by the method of Serrano, Gancedo & Gancedo (1973); 50 μl toluene/ethanol (1:4, v/v) was added to 50 mg wet wt of washed yeast in 1 ml 100 mM-phosphate buffer, pH 7·7, containing 200 mM-KCl and 10 mM-mercaptoethanol, and the mixture was agitated continuously for 5 min. The suspension was then used directly in enzyme assays employing cuvettes with a 2 mm light path because of the turbidity of the suspension.

Protein was released from toluene-lysed cells by passage through a French press and determined, as was that in cell-free extracts, by the biuret method (Gornall, Bardawill & David, 1949).

Enzyme assays. Phosphofructokinase [EC 2.7.1.11; ATP:D-fructose-6-phosphate 1-phosphotransferase] was initially assayed by the method of Sols & Salas (1966) and later by the method of Mazón *et al.* (1974). Both assays were coupled with added aldolase, triosephosphate isomerase and α-glycerophosphate dehydrogenase to the oxidation of NADH which was followed continuously at 340 nm.

Xylulose-5-phosphate phosphoketolase [EC 4.1.2.9; D-xylulose-5-phosphate D-glyceraldehyde-3-phosphate-lyase (phosphate-acetylating)] was assayed by method A of Goldberg, Fessenden & Racker (1966), in which the glyceraldehyde 3-phosphate being produced is converted to fructose 1,6-bisphosphate which is then estimated. This assay is discontinuous (see also Whitworth & Ratledge, 1977). Fructose-6-phosphate phosphoketolase [EC 4.1.2.22; D-fructose-6-phosphate D-erythrose-4-phosphate-lyase (phosphate-acetylating)] was assayed by method B of Goldberg *et al.* (1966), in which the acetyl phosphate produced is estimated as its ferric hydroxamate (see also Whitworth & Ratledge, 1977).

Phosphogluconate dehydratase [EC 4.2.1.12; 6-phospho-D-gluconate hydro-lyase] and phospho-2-keto-3-deoxy-gluconate aldolase [EC 4.1.2.14; 6-phospho-2-keto-3-deoxy-D-gluconate D-glyceraldehyde-3-phosphate-lyase] were assayed by the methods of Ng & Dawes (1973) in which the pyruvate formed is trapped and converted to the dinitrophenylhydrazone.

Acetate kinase [EC 2.7.2.1; ATP:acetate phosphotransferase] was assayed in the reverse direction using the method of Rose (1955) as modified by Whitworth & Ratledge (1977).

Phosphate acetyltransferase [EC 2.3.1.8; acetyl-CoA:orthophosphate acetyltransferase] was assayed by the method of Klotzsch (1969) by directly measuring the acetyl-CoA being formed.

Incorporation of radioactivity. The uptake of [1-^{14}C]glucose and [6-^{14}C]glucose into washed suspensions of *Candida* 107 and *S. cerevisiae* was followed by measuring $^{14}CO_2$ released and ^{14}C incorporated into lipid. Yeast suspension (3 ml; about 50 mg dry wt) in 50 mM-phosphate buffer, pH 7·0, containing 200 mM-$KHCO_3$, was placed in the main chamber of a sealed Warburg flask with the centre well containing a circle of Whatman glass-fibre paper saturated with 10 % (w/v) Hyamine hydroxide in methanol and the side-arm containing 5 M-H_2SO_4. Labelled glucose (100 μl; 2 mg; 10 μCi) was injected through the Suba-seal cap. After 60 min at 30 °C with continuous shaking, a sample (0·5 ml) was removed and immediately frozen in liquid nitrogen. Metabolism in the remaining cells was stopped by tipping in the acid. The glass-fibre paper was transferred to toluene-based scintillation fluid and counted by liquid scintillation to determine the total $^{14}CO_2$ evolved. The frozen sample was disrupted by passage through a French press and the lipid was extracted into 40 ml chloroform/methanol (2:1, v/v). The extract was washed once with 1 % (w/v) NaCl, twice with distilled water, dried over anhydrous $MgSO_4$, taken to dryness under vacuum, weighed and dissolved in 1 ml toluene; the radioactivity was determined by liquid scintillation.

Chemicals. Enzymes, coenzymes and substrates were from Sigma or Boehringer. Radiochemicals were obtained from The Radiochemical Centre, Amersham.

RESULTS AND DISCUSSION

Cell-free extracts prepared by conventional means (ultrasonication, passage through Hughes and French presses, and grinding freeze-dried cells with Ballotini beads) showed little or no activity of phosphofructokinase, no matter on which substrate the yeast had grown (glucose, sucrose, lactose, ethanol and alkanes were tried). There was no suggestion that this was due to inhibition of an otherwise active enzyme as even affinity chromatography of extracts using Cibacron Blue 3GA (see Tamaki & Hess, 1975) failed to elicit activity. Activity was found only after treating the yeast with toluene and assaying the resulting cells (Table 1). The assay methods of Mazón *et al.* (1974) and Sols & Salas (1966) both gave similar results and a specific activity of over 200 nmol substrate used min^{-1} (mg protein)$^{-1}$ was found in glucose-grown cells. When extracts of cells, prepared conven-

Table 1. *Activity of phosphofructokinase in Candida* 107 *made permeable with toluene*

Phosphofructokinase was assayed by the method of Mazón *et al.* (1974) with 2 mm path length cuvettes. Identical results were obtained by the method of Sols & Salas (1966). Maximum activity corresponds to 212 nmol substrate used min^{-1} (mg protein)$^{-1}$.

	ΔE_{340} min^{-1}
Complete assay	0·06
−aldolase	0·008
−aldolase and α-glycerophosphate dehydrogenase	0
−fructose 6-phosphate	0
−glucose 6-phosphate	0·04
+phosphoglucose isomerase	0·06
Complete system after passage through French press	0

Table 2. *Metabolism of radioactive glucoses by Candida* 107 *and S. cerevisiae*

	^{14}C incorporated into lipid [d.p.m. (mg dry wt lipid)$^{-1}$]		^{14}C recovered as $^{14}CO_2$ [d.p.m. (mg cell dry wt)$^{-1}$]	
Substrate	*Candida* 107	*S. cerevisiae*	*Candida* 107	*S. cerevisiae*
[1-^{14}C]glucose	519	937	313	258
[6-^{14}C]glucose	3163	2077	34	450

tionally, were added to the toluene-treated cells, there was no inhibition of activity. Furthermore, passage of toluene-treated cells through a French press destroyed all activity of phosphofructokinase and we thus conclude that previous inactivity had been due to the extreme lability of the enzyme.

This finding of phosphofructokinase reverses our previous conclusion that *Candida* 107 is devoid of the Embden–Meyerhof pathway (Whitworth & Ratledge, 1975*a*). The contribution of this pathway to glucose metabolism was determined by following the incorporation of radioactivity from [1-^{14}C]glucose and [6-^{14}C]glucose into washed suspensions of *Candida* 107 and, as a comparison, into *S. cerevisiae* (Table 2). Incorporation into lipid was followed as this gives an indirect measure of the formation of acetyl-CoA. The proportion of glucose which was metabolized via the pentose cycle (PC) was calculated from the formula of Wood, Katz & Landau (1963):

$$\frac{\text{Radioactivity in lipid from [1-}^{14}\text{C]glucose}}{\text{Radioactivity in lipid from [6-}^{14}\text{C]glucose}} = \frac{1-\text{PC}}{1+2\text{PC}}$$

as being about 63%, the remainder being metabolized via the Embden–Meyerhof pathway. With *S. cerevisiae* about 25% of glucose was metabolized via the pentose cycle which is consistent with other reports (Wang, 1972).

Respired $^{14}CO_2$ from the two radio-isomers of glucose was also monitored (Table 2) and confirmed that with *Candida* 107 most of the glucose was being metabolized via the pentose cycle, i.e. more ^{14}C was lost from [1-^{14}C]glucose than from [6-^{14}C]glucose. [Similar results to these have been obtained with *R. gracilis* by Höfer *et al.* (1971).] However, the release of $^{14}CO_2$ from [6-^{14}C]glucose with *S. cerevisiae* was higher than from [1-^{14}C]glucose. This result was unexpected but was obtained repeatedly.

If about 60% of the glucose was being metabolized by the pentose pathway, and the remainder by the Embden–Meyerhof pathway, this would still produce a situation where fewer C_2 units would be produced than indicated by the high molar growth yields of *Candida* 107 ($Y_{glucose}$ = 100; Gill *et al.*, 1977). The possibility that the pentose cycle was augmented with a supplementary pathway or enzyme was therefore investigated.

The Entner–Doudoroff pathway, which would diverge from 6-phosphogluconate and eventually lead to loss of CO_2 from the C-1 atom of glucose at the level of pyruvate

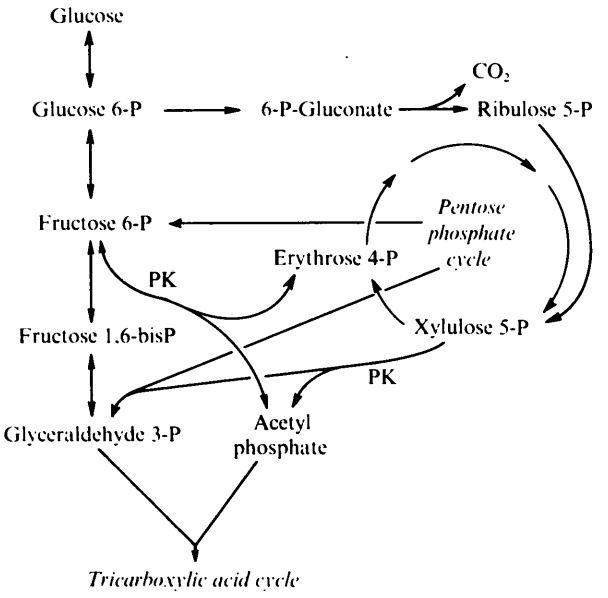

Fig. 1. Pathways of glucose metabolism in *Candida* 107. PK, Phosphoketolase.

dehydrogenase, was absent: the two key enzymes of this pathway, phosphogluconate dehydratase and phospho-2-keto-3-deoxy-gluconate aldolase, were not detected in extracts of *Candida* 107 grown on glucose though both were found in extracts of gluconate-grown *Escherichia coli* examined simultaneously.

The other alternative considered was phosphoketolase. This enzyme had been assumed by Höfer *et al.* (1971) to be present in *Rhodotorula gracilis* to account for glucose metabolism, though apparently no attempt was made to find it. Xylulose-5-phosphate phosphoketolase when assayed in extracts of *Candida* 107 grown on glucose in batch or continuous culture had an activity of only 2 nmol acetyl-CoA formed min^{-1} (mg protein)$^{-1}$ confirming the results of Whitworth & Ratledge (1977). In extracts of cells grown on *n*-alkanes, no activity of the enzyme was found. Activity of fructose-6-phosphate phosphoketolase in glucose-grown cells was likewise very low at 6 nmol acetyl-CoA formed min^{-1} (mg protein)$^{-1}$. However, due to the discontinuous method of assay and its relative insensitivity we were unable to determine the kinetics of the phosphoketolase, though Whitworth & Ratledge (1977) found that the enzyme in *R. graminis* had a K_m for xylulose 5-phosphate of 1·25 mM. Phosphoketolase (for both xylulose 5-phosphate and fructose 6-phosphate) was detected at about the same activities in cells made permeable with toluene and thus its low activity appeared to be genuine.

In keeping with the low activity of phosphoketolase we found only slight activity of enzymes which act on acetyl phosphate, the product of phosphoketolase action. In extracts of glucose-grown *Candida* 107, acetate kinase activity was 1 nmol acetyl phosphate used min^{-1} (mg protein)$^{-1}$ but phosphate acetyltransferase, the only other enzyme known to act on acetyl phosphate, was not detected.

Although the activity of phosphoketolase was low the most significant point was that it was there at all, having only previously been recorded in a few bacteria (see Whitworth & Ratledge, 1977). We assume therefore that this enzyme does make some contribution to glucose metabolism and will serve to increase the numbers of C_2 units being produced from glucose. The extent to which it competes with transketolase for the substrate, xylulose 5-phosphate, could not be determined.

We conclude that phosphofructokinase exists in *Candida* 107 but it is highly labile.

Glucose metabolism in this yeast thus occurs via the pentose cycle and the Embden–Meyerhof pathway with the former route predominating. Both pathways are supplemented to an unknown extent by phosphoketolases acting on xylulose 5-phosphate and fructose 6-phosphate (see Fig. 1).

The metabolism of glucose in Rhodotorula (Rhodosporidium) yeasts is probably similar. Phosphoketolase has been found in *R. graminis* and *R. glutinis* (Whitworth & Ratledge, 1977) which, like *Candida* 107, have been previously thought of as phosphofructokinase-deficient yeasts, and phosphofructokinase activity has now been found in *R. graminis* following treatment with toluene (P. A. Botham, unpublished work) which thus complements the discovery by Mazón *et al.* (1974) of an unusual phosphofructokinase in *R. glutinis*. Our previous view that lipid accumulated in these yeasts because metabolism was 'out of control' due to lack of phosphofructokinase (Whitworth & Ratledge, 1975*b*) clearly must be substantially revised. The reasons for lipid accumulation thus remain as obscure as before but the occurrence of phosphoketolase may not be irrelevant in this respect.

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