Balance of Production and Consumption of ATP in Ammonium-starved *Saccharomyces cerevisiae*

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To establish a balance between the ATP produced in catabolism and the ATP consumed in net biosynthesis of cellular components the energy metabolism of *Saccharomyces cerevisiae* utilizing glucose in the absence of a nitrogen source (resting cells) was studied. The following results were obtained. (i) Cell number and biomass increased 2- and 2.5-fold, respectively, during the first 8 h of ammonium starvation. After this period, both values remained constant. (ii) The rate of sugar consumption and ATP production decreased with the duration of starvation to about 20% of the original in 24 h. (iii) About 60% of the sugar consumed was fermented to ethanol and about 10% assimilated as cellular material. Of the assimilated sugar, as much as 80% was accumulated as carbohydrate. (iv) Only 15% of the total ATP produced in catabolism seems to be consumed in net biosynthesis and maintenance of intracellular pH. The fate of the remaining 85% is unknown.

INTRODUCTION

Knowledge on yeast energy metabolism has increased greatly over the years (Gancedo & Serrano, 1987) and yet several important questions remain unsolved (Lagunas, 1986). One of these is the fate of ATP produced in catabolism. Stouthamer (1973) calculated the amount of ATP required by yeast for biosynthesis of cellular material and the amount of ATP required for nutrient transport (Gancedo & Serrano, 1987). The balance between these values and the amount of ATP produced by growing yeast on different substrates suggested that only 40% of the total ATP is spent in these functions (Lagunas, 1976; Gancedo & Serrano, 1987). Similar results were reported in the case of bacteria (Hempfling & Mainzer, 1975) and it was proposed that the portion of ATP which is unaccounted for might be used in maintenance of the cellular structures and preservation of ionic composition (Stouthamer & Bettenhausen, 1973). However, the fact that the amount of ATP required to maintain viability of starved yeast (Lagunas, 1976) and bacteria (Stouthamer & Bettenhausen, 1975) was negligible suggested that the non-identified ATP-consuming functions were dependent on growth and/or active metabolism (Lagunas, 1976; Gancedo & Serrano, 1987). These hypotheses have not been proved and the fate of as much as 60% of the total ATP produced by micro-organisms is still unexplained (Lagunas, 1986; Gancedo & Serrano, 1987).

In this work the energy metabolism of yeast consuming glucose in the absence of a nitrogen source (resting cells) was studied. Our rationale was that detection of differences in the balance between production and consumption of ATP in growing versus resting yeast could help in the identification of ATP-consuming functions related to growth. Our results indicate that although important catabolic changes occur in the shift from growing to resting conditions, the balance of the production and utilization of ATP remained constant.

METHODS

Reagents. Enzymes and nucleotides were from Sigma. D-[U-¹⁴C]Glucose was from Amersham. All other reagents were of analytical grade.

Strain, media and culture conditions. *Saccharomyces cerevisiae* strain ATCC 42407 was grown aerobically in a synthetic medium as described previously (Lagunas, 1976) with 2% (w/v) glucose as carbon source. Cells growing exponentially (about 1 mg dry yeast ml⁻¹) were harvested by centrifugation, washed with ammonium-free medium (Lagunas, 1976) and suspended in this medium in the presence of 2% (w/v) glucose at about 0.3 mg dry cells ml⁻¹. The suspensions were incubated at 30 °C anaerobically as described previously (Lagunas, 1979).

Analytical procedures. Anaerobic fermentation was measured manometrically at 30 °C as described previously (Lagunas, 1979). Glucose metabolism was studied using D-[U-¹⁴C]glucose. Samples (5 ml) of yeast in ammonium-free medium, containing 2% (w/v) D-[U-¹⁴C]glucose [1 µCi mmol⁻¹ (37 kBq mmol⁻¹)] were placed in 50 ml flasks. The flasks were stoppered with rubber caps fitted with a windowed plastic tube containing 2 ml hyamine. Two hypodermic needles were passed through the caps. Nitrogen was bubbled through one needle and received after passage through the suspension in a test-tube containing 2 ml hyamine to trap the ¹⁴CO₂ produced by the cells. After 30 min incubation at 30 °C with vigorous shaking, the needles were removed. At various times, 0.1 ml 10 M-HCl was added to the cell suspension and ¹⁴CO₂ absorption allowed to continue for a further 30 min. Radioactivity incorporated into the cells was measured by filtering 0.5 ml of the yeast suspension through Whatman filters; after washing with 5 ml cold water, the filters were dried and the radioactivity counted. The radioactivity absorbed by hyamine, as well as that remaining in the medium, was determined. Glucose, ethanol, glycerol, acetaldehyde and acetate present in the media were determined by conventional enzymic methods (Lagunas, 1976) in samples filtered through Millipore filters.

Cell number was determined by plating on a solid medium containing 2% (w/v) glucose. Cell dry wt was determined as described previously (Lagunas, 1976). Carbohydrate (Herbert *et al.*, 1971), total protein (Jayaraman *et al.*, 1966), lipid (Lagunas & Gancedo, 1973) and DNA and RNA content of the cells (Schmidt & Thannhauser, 1945) were determined as described previously. Polyphosphate content was determined using a rapid sampling method that included washing the cells with methanol/water (50/50, v/v) at -40 °C (Sáez & Lagunas, 1976). This avoids contamination with orthophosphate present in the medium and hydrolysis of polyphosphate during sampling. Polyphosphate was extracted as described by Harold (1966) and hydrolysed by heating at 95 °C in the presence of 5 M-HCl. The resulting orthophosphate was determined as described by Bernhart & Wreath (1955).

RESULTS AND DISCUSSION

Contribution of different catabolic pathways to glucose utilization by ammonium-starved cells

Anaerobic conditions were used because, in the absence of oxygen, glycolysis is the only known pathway to produce ATP, and therefore the amount of ATP formed in catabolism can be calculated easily. Ethanol and glycerol production accounted for 66 and 8%, respectively, of the total sugar consumed whereas acetic fermentation accounted for only 1.5% (Table 1). Acetaldehyde was not detected. However, some excretion of this metabolite, followed by evaporation, seems likely as its boiling point is 20 °C and the experiments were done at 30 °C. In favour of this hypothesis is the excess of CO₂ recovered over two-carbon compounds that accumulated in the medium. This excess of CO₂ cannot be ascribed to operation of the pentose

Table 1. *Assimilation and fermentation products accumulated in the medium during catabolism of glucose by ammonium-starved yeast*

Yeast cells were grown aerobically, harvested during exponential growth, suspended in ammonium-free medium containing 2% (w/v) D-[U-¹⁴C]glucose [1 µCi mmol⁻¹ (37 kBq mmol⁻¹)] and incubated anaerobically. Values are means ± SD (eight experiments).

Duration of ammonium starvation (h)	Glucose consumed	Glucose assimilated	CO ₂	Ethanol	Glycerol	Acetaldehyde	Acetate
			[µmol (ml culture) ⁻¹]				
6	51 ± 7	4.5 ± 0.3	75 ± 7	66 ± 8	7.3 ± 0.9	<1.0	1.0 ± 0.3
24	72 ± 5	7.0 ± 0.05	125 ± 14	97 ± 12	11.8 ± 0.6	<1.0	3.2 ± 0.5

Fig. 1. Changes during ammonium starvation. Cells were grown aerobically, harvested during exponential growth, suspended in ammonium-free medium containing 2% (w/v) glucose and incubated anaerobically. Δ , Glucose assimilation; $\circ$, biomass; $\bullet$, cell number. Values are means $\pm$ SD of eight experiments.

Table 2. Content of cellular components

Cells were grown and treated as described in Table 1. Values are means $\pm$ SD (eight experiments).

Duration of ammonium starvation (h)	Yeast dry wt	Carbohydrate	Protein	Lipid	DNA	RNA	Polyphosphate
[$\mu\text{g (ml suspension)}^{-1}$]							
0	390	105 $\pm$ 15	168 $\pm$ 14	5	8 $\pm$ 3.6	52 $\pm$ 5.9	9 $\pm$ 2.5
6	649	259 $\pm$ 40	197 $\pm$ 41	14	8 $\pm$ 1.1	67 $\pm$ 7.0	18 $\pm$ 3.8
24	944	527 $\pm$ 59	183 $\pm$ 22	19	9 $\pm$ 0.8	64 $\pm$ 5.9	30 $\pm$ 8.8

phosphate pathway since the contribution of this pathway to glucose catabolism is quite small (Lagunas & Gancedo, 1973). The results suggest that only about 10% of the total sugar consumed was assimilated into cellular material.

A similar contribution of the different pathways to the one observed here with resting yeast has been reported with growing yeast (Lagunas, 1976), indicating that catabolism of glucose is similar under both conditions.

Changes during ammonium starvation

Fermentation of glucose by exponentially growing yeast occurred at about 40 mmol glucose (g protein) $^{-1}$ h $^{-1}$ (Fig. 1). However, as soon as these cells were transferred to a medium lacking a nitrogen source the fermentation rate dropped to 50% of the initial value, possibly as a consequence of the effect of ammonium ion on phosphofructokinase (Ramaiah, 1974). As ammonium starvation proceeded, the fermentation rate decreased progressively as a consequence of the irreversible inactivation of glucose transport in resting cells (Lagunas *et al.*, 1982).

Cell number increased about 2-fold during 4 h of ammonium starvation (Fig. 1). Other metabolic conditions in which cell division proceeds in the absence of a nutrient have been reported (Hartwell, 1974). The increase in cell number was accompanied by an assimilation of sugar that occurred at a constant rate for about 8 h. During this period, biomass increased about 2.5-fold. Afterwards, this parameter remained almost constant (Fig. 1).

Cellular composition changed during ammonium starvation as shown in Table 2.

Table 3. *Calculated ATP consumption in the net biosynthesis of the cellular components*

The values have been calculated, as described by Stouthamer (1973), from the data in Table 2. The following assumptions were made: polymerization of glucose to polysaccharide ($C_6H_{12}O_5$)_n requires 2 mol of ATP per monomer; synthesis of fatty acids ($C_{16}H_{32}O_2$) requires 16 mol of ATP mol⁻¹; synthesis and polymerization of RNA ($C_{10}N_5H_{14}O_6$)_n require 10 mol of ATP per monomer; transport of orthophosphate and polymerization to polyphosphate (PO_4H)_n require 2 mol of ATP per monomer.

Cellular component	ATP consumed (mmol per 100 mmol glucose utilized)	
	Ammonium starvation for 6 h	Ammonium starvation for 24 h
Carbohydrate	3.8	7.2
Lipid	0.8	0.9
RNA	1.0	0.6
Polyphosphate	0.6	1.0
Total	6.2	9.7

Carbohydrate content increased 5-fold in 24 h whereas lipid and polyphosphate content increased about 3-fold. Significant increments of other cellular components were not detected. These results indicate that as much as 80% of the assimilated sugar was accumulated by the cells as carbohydrate, most likely as glycogen and trehalose (Lillie & Pringle, 1980). They also indicate that whereas carbohydrate and protein content accounted for 26 and 41%, respectively, of the cellular material of growing yeast, they accounted for as much as 56 and 18% in resting yeast.

Balance of the production and utilization of ATP

Production of ATP in catabolism of glucose can be calculated since each mol of sugar transformed into ethanol and acetate produces a net gain of 2 mol of ATP, and a net expenditure of 2 mol of ATP takes place in the transformation of 1 mol of sugar to glycerol. On the other hand, an approximate estimation of the amount of ATP required for the formation of cellular material can also be done taking into account the amount of ATP spent in biosynthesis and polymerization of macromolecules (Stouthamer, 1973). According to these calculations, the data in Table 1 indicate that resting yeast produces about 120 mol of ATP per 100 mol of catabolized hexose. They also indicate that the biosynthesis of accumulated compounds from a similar amount of hexose requires 6 to 10 mol of ATP (Table 3). A comparison of both values suggests that, of the overall ATP produced in catabolism by resting yeast, only 10% is used in synthesis of cellular components the remainder being spent in functions other than net biosynthesis. One of these functions could be related to the maintenance of intracellular pH. In our experimental conditions the plasma membrane ATPase would be mainly responsible for the extrusion of H⁺ coming from passive influx from the medium, and from the acetic and succinic acid produced in catabolism (Gancedo & Serrano, 1987). Passive influx of H⁺ through the plasma membrane occurs at a rate of about 2 µmol (g dry wt)⁻¹ min⁻¹ (Leao & van Uden, 1984) and about 1% of glucose is metabolized to acetate (Table 1) and succinate (Gancedo & Serrano, 1987). These data and those in Table 1 suggest that of the 120 mol of ATP produced in the catabolism of 100 mol of glucose, only about 5 mol are spent in maintenance of the cellular pH. This assumes that all CO₂ produced in fermentation is excreted as gas without expenditure of ATP. However, the possibility that CO₂ is excreted as carbonic acid cannot be excluded, and, in this case, an enormous amount of ATP would be needed to maintain cellular pH. Our observations indicate that the proportion of energy spent in functions not directly related to biosynthesis is similar in growing and in resting yeast. The possibility that a high expenditure of ATP is required to excrete CO₂ in the form of carbonic acid should be seriously considered.

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