SHORT COMMUNICATION

Intracellular Concentrations of Citric and Isocitric Acids in Cultures of the Citric Acid-excreting Yeast *Saccharomycopsis lipolytica* Grown on Alkanes

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The intracellular concentrations of citric and isocitric acids were very low for most of the growth cycle of *Saccharomycopsis lipolytica* on alkanes. They increased linearly during the phase of acid excretion and the intracellular concentration was similar to the extracellular concentration (for citric acid) or a little below (for isocitric acid). The high ratios of isocitric to citric acid observed in the external medium (0·73) and in the internal pool (0·42) may be explained by compartmentalization of the metabolism of these acids which probably involves a selective transport of isocitric acid from the mitochondrion to the cytoplasm. The results suggest that acid excretion from the cytoplasm to the external medium takes place by diffusion.

INTRODUCTION

Extracellular production of citric and isocitric acids from yeasts, in particular from *Saccharomycopsis lipolytica* grown on alkanes, has been well documented (Nakanishi *et al.*, 1972; Hattori *et al.*, 1974). Production of acids usually begins after growth has stopped because of nitrogen limitation and is linear for over 50 h until metabolic activity decreases (Marchal *et al.*, 1977*a*). Also noteworthy are the large amounts of acids produced (which can exceed 200 g l^{-1}) and the high proportion of isocitric acid in the mixture (about 40%) when alkanes are used as the carbon source (Marchal *et al.*, 1977*a*; Aiba & Matsuoka, 1978; Tréton *et al.*, 1978). This proportion of isocitric to citric acid is far more than the 6·2% expected on the basis of the equilibrium constant for the reaction catalysed at pH 7·4 and 25 °C by aconitate hydratase (EC 4.2.1.3; Lowenstein, 1969). In order to obtain information on the mechanism of acid excretion, we have measured the intracellular concentration of citric and isocitric acids during the course of such a growth cycle.

METHODS

Cultures. Saccharomycopsis lipolytica 102 was grown in a fermenter as previously described (Marchal *et al.*, 1977*a*), except that hexadecane replaced the paraffin fraction as carbon source. The yeast was harvested by centrifuging (48000 *g* for 10 min at 4 °C) at various stages of the growth cycle. The yeast pellet was immediately used to determine the internal volume of the cells and the internal concentration of the acids. The supernatant was used to determine the concentration of acids in the culture medium.

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Determination of citric and isocitric acids. Citric and isocitric acids were determined separately in the culture medium and in the cell extracts. For citric acid, an enzymic method using citrate lyase was used (Dagley, 1974). An enzymic assay was also used for isocitric acid (Siebert, 1974).

Determination of the internal volume of the yeast cells. A method involving a radioactive polymer which cannot diffuse into the internal volume of the yeast cells was used. The radioactivity of 0·1 ml of a 80 μM aqueous solution of inulin-[^{14}C]carboxylic acid [10 mCi mmol^{-1} (370 MBq mmol^{-1}); The Radiochemical Centre, Amersham] was first measured in 20 ml of scintillation mixture (Bray, 1960). The same amount of inulin-[^{14}C]carboxylic acid solution was then added to a suspension of 2·5 g (wet wt) yeast pellet in 2·4 ml 50 mM-potassium phosphate buffer (pH 7·0). The mixture was vigorously shaken and centrifuged at 48000 g for 10 min. The radioactivity of 0·1 ml of the supernatant was again measured in 20 ml of Bray's mixture. These two determinations allowed the estimation of the external volume (V_e) of medium entrapped in a yeast pellet. The average value obtained for V_e was 0·32 ml (g wet wt)$^{-1}$. The water volume (V_w) of the pellet was obtained from the difference between wet weight and dry weight. A typical value for V_w was 0·75 ml (g wet wt)$^{-1}$. The internal volume (V_i) of the cells was calculated as: $V_i = V_w - V_e$ [average value: $0{\cdot}75 - 0{\cdot}32 = 0{\cdot}43$ ml (g wet wt)$^{-1}$].

Determination of the internal concentration of acids. A portion (2·5 g) of the unwashed yeast pellet was suspended in 5 ml water and the intracellular acids were extracted by placing the suspension in a boiling water bath for 15 min as described by Otsuka *et al.* (1965). No destruction of the acids was detected in controls submitted to the same treatment. It has also been verified that practically no change in the intracellular concentrations of the acids occurred during handling of the yeast prior to extraction (a decrease of 4% in isocitric acid and no variation in citric acid were observed when the yeast pellet was maintained at room temperature for 1 h). Determination of the acids in the extracts allowed the calculation of their internal concentrations in the cells, using the values obtained for V_i, V_e and for the external concentrations of both acids. This method obviated the need to wash the cells before extraction, thus avoiding the possibility (see below) that a portion of the internal acids could be removed by repeated washings.

RESULTS AND DISCUSSION

Figure 1 illustrates the two phases of growth and of acid excretion which characterize the fermentation of alkanes by *S. lipolytica* in a nitrogen-limited medium. The rate of isocitric acid excretion was 73% of that of citric acid. The internal concentrations of both acids were below the limit of detection during most of the growth phase but then they began to rise, the concentration of citric acid increasing first before the end of growth and before the excretion of acids began. During the phase of excretion, the internal concentrations of both acids increased linearly and were lower (in the case of isocitric acid) or of the same magnitude (in the case of citric acid) as the concentration of the acids in the culture medium. The high ratio (0·42 at 80 h) of isocitric to citric acid in the internal pool is noteworthy in view of the fact that isocitric acid originates only from citric acid. These results suggest that the acids are held in different cellular compartments, in agreement with the results of studies on the localization of the enzymes involved in their metabolism. According to present views, in *S. lipolytica*, as in other eukaryotes, citrate and isocitrate are generated in the mitochondrion where they can be oxidized through the tricarboxylic acid cycle. However, a portion of isocitric acid is transported to the cytoplasm where it is either converted to glutamate or metabolized through the glyoxylate cycle, the latter sequence taking place inside organelles called 'microbodies' (Aiba & Matsuoka, 1978). This metabolism suggests the existence of a selective system for transporting isocitric acid from the mitochondrion to the cytoplasm where it can then accumulate in concentrations greater than that expected on the basis of the thermodynamic equilibrium constant for citric and isocitric acids. The overall internal concentration of both acids rises when biosynthesis stops because of nitrogen limitation; excretion into the external medium then occurs. Passive diffusion is a likely mechanism for this process because the respective external and internal concentrations of each acid remain of the same order of magnitude during the phase of acid excretion. It is assumed that the concentration of isocitric acid in the cytoplasm is greater than that of the entire cell and is at least as high as the external concentration. In support of such a mechanism, lower intracellular concentrations of acids were detected in cells which had been

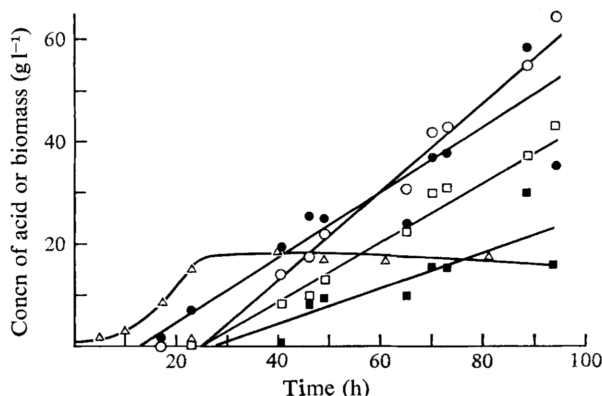

Fig. 1. Variations of intracellular and extracellular concentrations of citric and isocitric acids during growth of *Saccharomycopsis lipolytica* on hexadecane in a fermenter. Extracellular (○) and intracellular (●) concentrations of citric acid; extracellular (□) and intracellular (■) concentrations of isocitric acid; growth (△).

washed four times in 50 mM-potassium phosphate buffer (pH 7·0) (33 mM for citric acid and 1 mM for isocitric acid in a sample taken at 65 h compared with 147 and 47 mM, respectively, in unwashed cells). Thus, some of the acids, in particular isocitric acid, may be extracted by the washing procedure. This probably explains why a lower proportion of isocitric acid in the internal pool of alkane-grown *S. lipolytica* was reported by Tréton *et al.* (1978).

The present interpretation allows us to suggest that the transport activity of isocitric acid from the mitochondrion to the cytoplasm will be much lower during growth on a carbon source which does not require the glyoxylate cycle to be active. This will result in a lower proportion of isocitric acid in the mixture of acids excreted. Glucose constitutes such a carbon source as shown by the absence of isocitrate lyase activity in glucose-grown cells of *S. lipolytica* (Marchal *et al.*, 1977*b*) and a consistently lower proportion of isocitric acid (usually about 10%) has been observed in the mixture of acids excreted after growth on this carbon source (Briffaut, 1978; Tréton *et al.*, 1978). The latter authors also reported a similar result using glycerol as carbon source.

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