Regulation of Isocitrate Lyase Level in Yeast Growing on External Carbon Sources or on Lipid Reserves[1]

Dependence on environmental conditions of isocitrate lyase (E.C.4.1.3.1) level in microorganisms is known[2] and is currently interpreted with reference to anaplerotic function of this enzyme operating in the glyoxylate cycle[3]. The adjustment of enzyme level to the metabolic state of the cells has been evidenced by cultivating microorganisms on various nutrients[2-5], or by studying selected mutants with deficiencies in metabolic pathways[6]. The results of the present work show that a net synthesis of isocitrate lyase occurs also when a suitable strain of yeast, capable of lipid storage in the cell, is cultivated in a mineral medium and has as sole carbon source its internal reserves. The presence of external glucose has a strong repressive effect: such a catabolite repression[7] is the most evident regulatory device for the system.

Material and methods. A strain of *Rhodotorula gracilis* was used. Lipid reserve-containing cells were obtained in the following way: agar-malt slant cultures, 24 h old, were transferred into a minimal mineral-ammonium medium[8], containing the required growth factors (thiamine and pantothenate) and 200 m*M* glucose as carbon source. After 24 h at 30 °C, with eccentrical rotative agitation, the cells were transferred into fresh mineral-ammonium medium + growth factors + glucose for 72–96 h at 30 °C. Stationary lipid-rich cells were formed and used as starting material for experiments. Other experiments with *Rhodotorula* growing on external nutrients were carried out by using directly cells from agar malt cultures.

For enzyme assay, the yeast cells were harvested by centrifugation and suspended 1:5 (w/v) in 100 m*M* phosphate buffer, containing 10 m*M* mercaptoethanol and 10 m*M* $MgSO_4$, at pH 7.4. The cells were disrupted in a Braun glass-beads homogenizer[9] (5 g of beads/g of yeast; 30 sec at 70 rev/sec) and a clear extract was obtained by centrifugation at 20,000 *g* for 20 min.

Isocitrate lyase activity was measured by the optical phenylhydrazine test of DIXON and KORNBERG[10], at 324 nm and 30 °C. For some experiments the modifications proposed by KORNBERG[11] were also introduced: no difference was noted in our conditions between the 2 procedures.

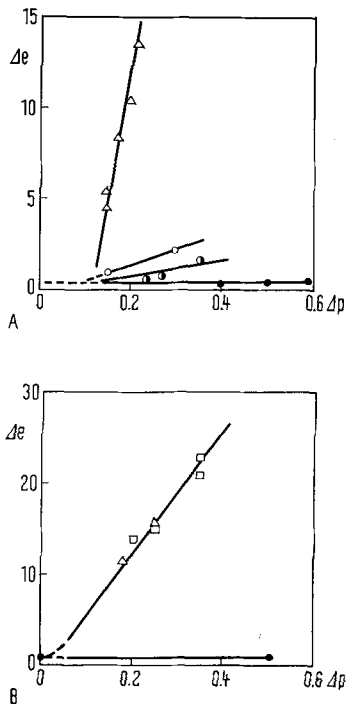
Respiratory quotient of yeast cells was determined by measuring the rate of O_2 consumption and CO_2 production by conventional Warburg technique.

To evaluate total protein synthesis, in experiments with cycloheximide and puromycin, total uptake into yeast cells and incorporation into proteins of leucine-1-C^{14} were measured by a scintillation technique, with a Tricarb Packard apparatus (80% efficiency). Proteins were precipitated according to SIEKIEVITZ[12].

Total proteins were determined by a biuret method, as described by BEISENHERZ et al.[13].

Results. Figure A shows a MONOD plot[14] of isocitrate lyase formation in cultures of *Rhodotorula* growing on glucose or on ethanol. By such a plot the enzyme formation is quantitatively expressed as the differential rate of the enzyme appearance in the growing culture, with respect to the total protein formation. The value of differential rate varies from about 1 in presence of 200 m*M* glucose to 140 in presence of ethanol: decreasing concentrations of glucose correspond to intermediate rate values. When the cells are grown in presence of both glucose and ethanol, the repressive effect of glucose is largely preponderant (Table I).

Lipid-rich yeast cells, in absence of any external carbon source, form isocitrate lyase with a high differential rate, not as high however as that attained by cells growing on ethanol (Figure B). The addition of



Differential rate of isocitrate lyase formation in *R. gracilis*. Enzyme formation (Δe) is plotted versus total protein formation (Δp). Δe is expressed as enzyme activity (nmoles of transformed substrate/min) per ml of yeast culture; Δp as mg of formed proteins per ml of culture. (A) *R. gracilis* growing in: (●) 200 m*M* glucose; (◑) 20 m*M* glucose; (○) 10 m*M* glucose; (△) 50 m*M* ethanol. (B) *R. gracilis* grown for 72 h in 200 m*M* glucose (stationary lipid-rich cells) and then transferred into the same volume of fresh mineral-ammonium medium containing: (●) 200 m*M* glucose; (△) 50 m*M* ethanol; (□) no carbon source.

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50 mM ethanol to the system does not change the rate of enzyme formation, whereas the repressive effect of glucose is also in this case very evident. 20 mM D-2-deoxyglucose is as effective as glucose in enzyme repression.

Under the same conditions as in experiments of the Figure, the respiratory quotient of yeast cells utilizing glucose, ethanol or internal reserves is the following: with glucose (5; 100 or 200 mM) : 1.1, with ethanol (100 mM) : 0.5, with no external C source: 0.5.

Table I. Effect of ethanol + glucose on the formation of isocitrate lyase in *R. gracilis*

Carbon source	Specific activity of isocitrate lyase (nmoles/min · mg protein)
50 mM ethanol	53.0
50 mM ethanol + 20 mM glucose	2.9
50 mM ethanol + 50 mM glucose	1.2
20 mM glucose	3.3
50 mM glucose	0.9

Enzyme formation is measured as specific activity in cells from agar-malt culture, cultivated 24 h in presence of the indicated carbon source.

Table II. Effect of antibiotics and amino acid analogs on the formation of isocitrate lyase in *R. gracilis*

Addition	Specific activity of isocitrate lyase (nmoles/min · mg protein)
–	26.5
70 μM cycloheximide	3.8
1 mM puromycin	21.5
10 mM DL-*p*-fluorophenylalanine	3.1
10 mM DL-azatryptophan	3.7
10 mM DL-ethionine	14.0

Enzyme formation is measured as specific activity in lipid-rich cells, cultivated 5 h in mineral-ammonium medium.

Table III. Effect of cycloheximide or puromycin on the rate of incorporation of leucine-1-C^{14} in total proteins of *R. gracilis*

Addition	Leucine-1-C^{14} incorporation in cell proteins (cpm/ml of cell suspension)
–	22,000
5.4 μM cycloheximide	4,200
18 μM cycloheximide	1,300
54 μM cycloheximide	900
0.5 mM puromycin	25,000
1 mM puromycin	24,000

A 10-min pulse (0.1 μC) was given to lipid-rich cells, cultivated 2 h in 200 mM glucose. Total uptake in the cells was constant, with values around 30,000 cpm/ml of cell suspension.

The data reported in Tables II and III indicate that the formation of isocitrate lyase requires de novo protein synthesis: the appearance of enzyme is strongly depressed by cycloheximide, a well-known inhibitor of protein synthesis at ribosomial level, as well as by amino acid analogs *p*-fluorophenylalanine and azatryptophan. Puromycin, another well-known inhibitor of protein synthesis, and ethionine, the methionine analog, inhibit to a lesser extent. Scant response to puromycin does not concern only specific formation of isocitrate lyase: tests of leucine-1-C^{14} incorporation into total proteins in presence of cycloheximide or puromycin show that in the *Rhodotorula* strain used for present experiments only the former has actually an inhibitory effect on protein synthesis (Table III).

Discussion. Results clearly show that lipid-rich cells of *Rhodotorula*, suspended in a medium with no external carbon source, form isocitrate lyase at a relevant rate, comparable in order of magnitude to that attained during growth on a C_2 compound. The parallelism between the behaviour of the lipid-rich cells and the cells growing on ethanol suggests, under the 2 conditions, the existence of a similar metabolic pattern favouring enzyme synthesis. The presence of external glucose modifies the pattern toward a repressive state in both cases. There is no enhancement by ethanol of isocitrate lyase synthesis in lipid-rich cells, and ethanol does not counterbalance the repressive effect of glucose. According to these data, an induction effect by ethanol may therefore be excluded; but of course there is no concluding evidence against any possible induction mechanism in isocitrate lyase control by metabolic products preceding or even following the isocitrate lyase reaction.

Until now, however, catabolite repression appears in *Rhodotorula* the only evident regulatory mechanism for the control of cellular level of isocitrate lyase. The identity of the responsible catabolite is still obscure: C_6 compounds closely related to glucose, or their UDP derivatives, are among the suspected substances[5,6,15–17]. The fact that one of these compounds – 6-phosphogluconate – is an inhibitor of enzyme activity[18] may suggest, especially for this metabolite, a role in the repression mechanism. Research is in progress on this point.

Riassunto. Quando il lievito *Rhodotorula gracilis* cresce sulle proprie riserve lipidiche si registra un aumento dell'enzima isocitrato liasi, fino ad un livello paragonabile come ordine di grandezza a quello raggiunto quando il lievito cresce su etanolo. Il glucosio reprime vigorosamente la sintesi dell'enzima, anche in presenza di etanolo. Dai dati finora noti un effetto induttivo dell'etanolo sembra da escludere, e la regolazione del livello di isocitrato liasi appare basata su meccanismi di repressione da cataboliti.

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