A Characteristic Pattern of Respiratory Enzymes in Cytoplasmic 'Petite' Strains of *Saccharomyces cerevisiae* as Revealed by Multiple Enzyme Analysis

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A system of 'multiple enzyme analysis' has been developed by adapting the Technicon Autoanalyzer so that groups of enzymes may be assayed automatically (Roodyn, 1965*a,b*). In particular, a system for assaying respiratory enzymes in yeast that reduce $K_3Fe(CN)_6$ at neutral pH has been established (Roodyn, 1965*b*). The criteria usually employed for the recognition of respiratory deficiency (the 'petite' mutation) in yeast are colony size, growth requirements (e.g. ability to grow on fermentable substrates but not on non-fermentable substrates; Ephrussi, 1953; Slonimski, 1953) and colony appearance (e.g. Horn & Wilkie, 1967). Such criteria, however, are relatively crude and may not detect more subtle differences between respiratory-deficient mutants. The application of a standard automated procedure to the study of respiratory enzymes in these mutants may therefore be of use.

Normal haploid and diploid marked strains from our laboratory stocks were used and spontaneous petite mutants isolated from strains 45 and 4C1 (45*sp* and 4C1*sp* respectively). Strain 4C1*sp*/Co was a spontaneous petite strain with a relatively high level of resistance to $CoSO_4$ (see Horn & Wilkie, 1967). The spontaneous rate of mutation to petite is approximately 4×10^{-3} in strains 45 and 188 and about 10^{-1} in strain 4C1. Petite strain 188*uv* was isolated as a single colony after u.v. irradiation of a suspension of cells of strain 188 and plating on yeast-extract-peptone (YEP)-agar. The frequency of petite colonies was around 12% in this case giving a 30-fold increase over the spontaneous rate. The acriflavine-induced petite 45*ac* was also isolated as a single colony from a plating of cells of strain 45 previously grown on a YEP-glucose medium containing 20 p.p.m. of acriflavine. This treatment resulted in an approximate 100-fold increase in petite frequency over that of non-treated cell (see Ephrussi, 1953).

Colonies were identified as petite by their appearance and response to the tetrazolium chloride overlay technique of Ogur, St John & Nagai (1957). Confirmation of respiratory deficiency was obtained by failure to grow on non-fermentable substrate (YEP-4% glycerol).

Petite strains were shown to be of the cytoplasmic

type (ρ -) by analysing crosses between them and normal strains, the criterion being failure of the petite character to reappear among the ascospores.

Diploid cultures obtained by crossing 45*ac* by 188*uv* and 45*sp* by 4C1*sp* were also analysed.

The cells were grown to stationary phase in YEP-2% glucose at 30° with vigorous shaking. The harvested cells were washed three times with distilled water, and 10g. of cells was mixed with 20ml. of 0.3M-mannitol and 30ml. of no. 12 ballotini, and disrupted in a Braun shaker, with 1 min. of fast shaking. The homogenate was centrifuged at 500*g* for 5 min. to remove glass beads, intact cells and cell debris, and the supernatant diluted in a suitable volume of 0.3M-mannitol and used for the enzyme assay.

Enzyme assays were carried out in a Technicon Autoanalyzer with a double manifold designed to give two incubation times, 6.4 min. and 11.6 min. (cf. Fig. 7 in Roodyn, 1965*b*). Substrates were placed in suitable repeating sequence on the sampler table, with a cup of distilled water between each substrate to allow for washing between samples. The sampler was run at 40 cups/hr. and the substrates were aspirated into a continuous stream of buffer, ferri-cyanide, neutral detergent and yeast homogenate. All assays were at 37° with O_2 -free N_2 as the gas phase. The final composition of the reaction mixture was as follows: potassium phosphate (25mM); Triton X-100 (0.05%, v/v); $K_3Fe(CN)_6$ (0.4mM); mannitol (0.1M); protein (0.50 μ g. of protein N/ml.); the final concentration of the various substrates was: NADH (0.18mM); sodium L(+)-lactate (1.8mM); sodium D(-)-lactate (1.8mM); potassium succinate (1.8mM). The pH of the reaction mixture was 7.4 and was monitored by a continuous-flow glass electrode. The reaction was followed by determining the fall in E_{420} caused by the reduction of the ferri-cyanide. The concentration of homogenate in the reaction mixture was increased in a linear fashion during the assay by using a gradient-making attachment (Fig. 1 in Roodyn, 1965*b*) and the enzyme activity was plotted against protein concentration in each case. The initial slope of this plot was used to calculate the enzyme rates, which

Table 1. *Ferricyanide reductases of normal and cytoplasmic petite strains of Saccharomyces cerevisiae*

Assay conditions are given in the text.

Strain	Ferricyanide reductase (μ moles/min./mg. of protein N)			
	NADH-	L(+)-Lactate-	D(-)-Lactate-	Succinate-
A: normal strains				
4Cl	2.64	2.09	0.02	0.01
10	4.81	0.97	0	0.10
21	4.20	0.60	0	0.06
40	0.95	0.17	0.33	0.01
45	2.96	1.40	0.10	0.15
188	1.80	0.35	0.38	0.01
X1 (diploid)	2.90	1.78	0.01	0.06
40 $\times$ 41 (diploid)	3.92	1.74	0.01	0.15
Average	3.02	1.14	0.11	0.07
B: cytoplasmic petite strains				
45 <i>sp</i>	2.42	0.15	0.80	0.02
45 <i>ac</i>	2.03	0.03	0.73	0
4Cl <i>sp</i>	1.30	0.09	0.13	0
4Cl <i>sp</i> /Co	1.42	0.01	0.39	0
188 <i>uv</i>	0.94	0	0.60	0
45 <i>ac</i> $\times$ 188 <i>uv</i> (diploid)	1.57	0.04	0.52	0
45 <i>sp</i> $\times$ 4Cl <i>sp</i> (diploid)	1.00	0	0.22	0
Average	1.53	0.05	0.48	0.003
$\frac{\text{Average B}}{\text{Average A}} \times 100$	51	4.4	436	4.2

are expressed as μ moles of ferricyanide reduced/min./mg. of protein N.

The results of the enzyme assays for NADH-, L(+)-lactate-, D(-)-lactate- and succinate-ferricyanide reductases are given in Table 1. Although there are differences between the various strains in the precise enzyme activities, two characteristic patterns clearly emerge. These are given by the average values for the normal strains (where the four enzymes are in the approximate proportions 3:1:0.1:0.1) and for the petite strains (where the proportions are approximately 1.5:0.05:0.5:0.005). Thus, on average, the formation of cytoplasmic petites results in a 50% fall in the activities of NADH-ferricyanide reductase, a 95% fall in the activities of L(+)-lactate- and succinate-ferricyanide reductases and a fourfold increase in the activity of D(-)-lactate-ferricyanide reductase. It may be seen from Table 1 that, for every cytoplasmic petite studied, the NADH-, L(+)-lactate- and succinate-ferricyanide reductase activities were well below the average value for the normal strains. Conversely, all the petites had higher D(-)-lactate-ferricyanide reductase activities than the normal.

These results suggest that a closely similar, if not identical, mutational change has occurred in all the petite strains, although they were obtained by very

different means. This possibility is further evidenced by the genetic and enzymic analysis of crossing various petite strains. In the crosses *sp* (spontaneous petite) $\times$ *sp* and *ac* (acriflavine-induced petite) $\times$ *uv* (u.v.-induced petite), all derived diploids were petite, and no reversions to respiratory competence were seen in selective platings on YEP-4% glycerol, in which about 10^8 cells were sampled in each case. Also, the enzyme patterns of these crosses were similar to the average 'petite' pattern (Table 1). There is thus no complementation at the biochemical level, nor restoration by genetic recombination.

The most striking changes are those observed with the lactate-ferricyanide reductases. The lactate dehydrogenase system of yeast is complex (Labeyrie & Slonimski, 1964) and it is important to distinguish between studies with ferricyanide and those with cytochrome *c* as electron acceptor (cf. Mahler, Mackler, Grandchamp & Slonimski, 1964). Nevertheless, results similar to ours have been reported by Sherman & Slonimski (1964) in their analysis of a cytoplasmic petite by using a cytochrome *c* system. In view of the consistent, and sometimes dramatic, fall in L(+)-lactate-ferricyanide reductase activity in the petite strains, and the consistent rise in the corresponding D(-)-lactate-ferricyanide reductase found in our experi-

ments, it is possible that a high ratio of D- to L-lactate-ferricyanide reductases may be a convenient marker for respiratory deficiency due to cytoplasmic mutation. More work is required to delineate precisely the changes that have occurred in the lactate dehydrogenase system. Also, it is not known at the moment whether the quantitative differences in enzyme activity of the various petite strains shown in Table 1 are due to physiological variations during the growth cycle, or reflect more fundamental genetic differences.

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The Relation of Hydrogen Exchange between Deoxyadenosylcobalamin and Water to the Role of the Coenzyme in Ribonucleotide Reduction

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5'-Deoxyadenosylcobalamin (coenzyme B₁₂) is required as a cofactor for the enzymic reduction of ribonucleotides to deoxyribonucleotides by cell-free extracts of *Lactobacillus leichmannii* (Blakley & Barker, 1964). Experiments with the partially purified enzyme have established that a major difference between the cobamide-dependent ribonucleotide reductase and a number of other cobamide-requiring enzymes such as glutamate mutase, methylmalonyl-CoA mutase and diol dehydrase is that in the reductase reaction solvent hydrogen atoms are incorporated into one of the products. Thus when the enzymic reduction of ATP is carried out in the presence of [³H]water, tritium is incorporated into dATP (Blakley, Ghambeer, Batterham & Brownson, 1966). To elucidate the role of deoxyadenosylcobalamin in the ribonucleotide reductase reaction of *L. leichmannii* we have now studied the exchange of hydrogen atoms between water and deoxyadenosylcobalamin in the presence of the ribonucleotide reductase.

Extracts of *L. leichmannii* grown under optimum conditions for the production of ribonucleotide reductase (Ghambeer & Blakley, 1966) were

prepared by the use of the Ribi pressure cell. After purification by protamine sulphate treatment, (NH₄)₂SO₄ fractionation and gel filtration, the reductase had a specific activity of 68 μmoles of ATP reduced/mg. of protein/hr. at 37° under standard conditions. Deoxy[5'-³H]adenosylcobalamin was synthesized as described by Frey & Abeles (1966), purified by column chromatography and crystallized from aqueous acetone (Barker, Smyth & Hogenkamp, 1963); the product had a specific activity of 630 000 counts/min./μmole. dITP was prepared by chemical deamination of dATP according to the method of MacNutt (1952).

A reaction mixture containing dimethylglutarate buffer, pH 7.3 (0.3M), dihydrolipoate (30mM), EDTA (1mM), deoxyadenosylcobalamin (0.8mM), ATP (0.5mM), enzyme (0.54mg.) and [³H]water (0.25c) in a total volume of 5ml. was incubated in the dark at 33°. After 1hr. deoxyadenosylcobalamin was isolated from the reaction mixture by phenol extraction, and re-extracted from the phenol into water by the method of Barker *et al.* (1963). Radioactivity measurements in a Packard Tri-Carb model 3003 liquid-scintillation spectrometer