Respiratory System of Rhodotorula glutinis
I. Inhibitor Tolerance and Cytochrome Components

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Summary. Oxygen uptake by the carotenoid-containing yeast, Rhodotorula glutinis, was not affected by concentrations of cyanide and antimycin A which completely inhibit the respiration of Saccharomyces cerevisiae. The tolerance of R. glutinis to these inhibitors was somewhat dependent on the age of the cultures. Reduced minus aerated difference spectra of cells revealed spectral changes presumably due to cytochromes and carotenoids. The kinetics of these spectral changes induced by oxygen were followed. Carotenoid deficient cells were prepared by growth in the presence of diphenylamine. Difference spectra of these cells revealed the presence of flavoprotein, and a, b, and c type cytochromes. Growth of R. glutinis was completely inhibited by concentrations of cyanide which did not affect respiration. Oxidation of reduced nicotinamide adenine dinucleotide by sub-cellular fractions was sensitive to cyanide and antimycin A. Although respiration of intact cells is tolerant to these inhibitors, studies with cell-free extracts suggest the presence of a cyanide and antimycin A-sensitive, cytochrome-linked, respiratory chain.

During the course of cytological investigations of the pigmented aerobic yeast Rhodotorula glutinis, numerous well-defined mitochondria were observed (17). Since the yeast cell is one of the simplest in which the existence of mitochondria has been demonstrated, a study of the ability of the mitochondria to catalyze terminal oxidations was initiated. The cytochrome-cytochrome oxidase system functions as the terminal oxidase in all aerobically-grown yeast examined to date (1, 6, 9, 10, 12).

Our results, however, indicate that respiration of intact cells of R. glutinis is tolerant to high concentrations of cyanide and antimycin A, suggesting that this yeast does not respire by the cyanide and antimycin A sensitive pathways which operate in mammalian tissues and yeast such as Saccharomyces cerevisiae. We, therefore, examined some of the general properties of the respiratory system of R. glutinis.

Materials and Methods

Cultures of R. glutinis were grown with vigorous aeration at 26 to 28°C in 500 ml flasks containing 100 ml of a glucose, ammonium sulfate, asparagine medium (13). The inoculum for the main culture was grown in a complex medium (8) with a final glucose concentration of 2%. This medium was also used in the cultivation of S. cerevisiae.

Cells were collected by centrifugation and washed 3 times with 0.001 M phosphate buffer, pH 7.0, and subsequently suspended in 0.2 M phosphate buffer, pH 7.0.

Oxygen uptake was measured either in a Warburg apparatus or polarographically; using the Clark oxygen electrode (3); both procedures yielded equivalent rates. Spectral data were obtained with a Cary Model 14 recording spectrophotometer equipped with a sensitive slide wire (full scale deflection, 0.1 OD unit) and a high intensity light source.

Antimycin A (crystalline Type III) was purchased from Sigma Chemical Company; cyanide was neutralized with potassium hydroxide prior to addition to the reaction mixture.

Details on cell rupture and fractionation of cell-free extracts are given in the text.

Effect of Cyanide and Antimycin A on the Respiration of R. glutinis and S. cerevisiae. The Q₁₀ values obtained for whole cells of R. glutinis

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are much lower than those of *S. cerevisiae* (table I). The respiration of *S. cerevisiae* is strongly inhibited by cyanide and antimycin A, whereas *R. glutinis* is relatively insensitive to these respiratory chain inhibitors. The effect of various concentrations of cyanide on respiration of cells obtained from cultures of different age is shown in figure 1. These data show that respiration of *S. cerevisiae* is sensitive to cyanide regardless of age, whereas tolerance to cyanide by cultures of *R. glutinis* is affected by the age of the culture, older cells being more resistant than younger cells. As indicated, low concentrations of cyanide resulted in a slight stimulation of respiration of *R. glutinis*.

**Absolute Spectrum of Intact Cells.** The absorption spectrum of *R. glutinis* (fig 2, curve A) was obtained by using the opal glass technique of Shibata (14). Major peaks were present at 472, 502 and 534 μm indicating the presence of carotenoid pigments. The Soret bands of the cytochromes, if present, seem to be masked by absorption due to carotenoids.

**Difference Spectrum of Intact Cells.** In order to determine what kinds of respiratory pigments were present in this yeast, reduced minus oxidized difference spectra of dense suspensions of intact cells were obtained. Cell suspensions were reduced by hydrosulfite or by O2 depletion due to endogenous respiration; the spectra are shown in figure 3. Absorption peaks at 560, 520, 485, 444, 417 μm and a shoulder at 603 μm were observed in samples reduced by endogenous respiration. When hydrosulfite was used for reduction, the band at 417 μm shifted to 428 μm. The presence of cytochrome a2 (peaks at 444 and 603 μm) and a c-type cyto-

### Table I. Effect of Cyanide and Antimycin A on Oxygen Uptake by *R. glutinis* and *S. cerevisiae*

<table>
<thead>
<tr>
<th></th>
<th>Oxygen Uptake/mg dry weight/hr at 24°C</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose 0.067 M</td>
<td>Glucose omitted</td>
</tr>
<tr>
<td><em>Rhodotorula</em> glutinis</td>
<td>17.8</td>
<td>13.3</td>
</tr>
<tr>
<td><em>Saccharomyces</em> cerevisiae</td>
<td>74.8</td>
<td>47.0</td>
</tr>
</tbody>
</table>

* 4 Day old cultures were used.
** O2 = μl O2 uptake/mg dry weight/hr at 24°C in pH 7.0, 0.1 M phosphate buffer; O2 uptake was measured polarographically.
*** Inhibition determined in the presence of glucose, and is expressed as the percent inhibition of total respiration.
The concentration of carotenoids, therefore, diphenylamine (DPA) was added to the growth medium in an attempt to inhibit carotenoid formation (5, 7). At a final concentration of $1 \times 10^{-4}$M, growth was not inhibited, but the cells were nearly colorless, indicating an inhibition in carotenoid formation. The absolute spectrum of these cells is shown in figure 2 (line C); these cells are referred to as DPA cells. If a concentration of $3 \times 10^{-3}$M DPA was used, somewhat yellowish cells resulted. The absolute spectrum of such cells is shown in figure 2 (line B). The absorption at 420 $\mu m$ in figure 2.

**Fig. 3.** Difference spectrum of *R. glutinis*. The cell suspensions were reduced by endogenous respiration or by hydrosulfite; the control cuvette was oxidized by vigorous shaking for 2 minutes. The concentration of cells was $6.8 \text{ mg dry wt/ml}$.

Chromatophores are suggested by these spectra. The peak at 417 $\mu m$ is probably due to the Soret peak of cytochrome $c$. The peaks at 560, 520 and 485 $\mu m$ are not typical of cytochrome bands and are probably due to carotenoids which undergo spectral changes upon aeration of the reference cuvette. Such peaks were not present in the carotenoid deficient cells prepared by growth in the presence of diphenylamine (see below). If the peaks at 560, 520 and 485 $\mu m$ are due to cytochromes, the absorption bands in the Soret region would be greater than observed.

**Time Course of Absorbancy Changes.** The absorbancy changes of the various components in difference spectra were followed by allowing both the sample and reference cuvette to be reduced endogenously. One of the suspensions was then aerated and used in the reference cuvette. The time course of the absorbancy changes is shown in figure 4.

The rapid absorbancy change at 444 $\mu m$ is presumably due to reduction of cytochrome $a_2$; parallel observations with the $O_2$ electrode showed that the $O_2$ was depleted at this time. The optical density at 460 $\mu m$ began to increase after the removal of oxygen, suggesting the involvement of flavoprotein. The changes at 417 $\mu m$, presumably due to cytochrome $c$, were difficult to interpret; these changes at 417 $\mu m$ were also observed in carotenoid deficient cells.

The velocity of the absorbancy changes at 560 $\mu m$, presumably due to carotenoids, was comparable to that of the cytochromes. It was also observed that the troughs in the difference spectrum (fig 3) at 470, 500 and 538 $\mu m$ became more pronounced with repeated reduction and re-oxidation of the reference cuvette. The disappearances of these troughs on standing was much slower than that of the 560 $\mu m$ component. These spectral changes are similar in some respects to the spectral changes produced in *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides* by oxygenation or illumination (4, 11, 15, 16). Further experiments will be required before the exact nature of these spectral changes in this yeast will be understood. In agreement with the results obtained with *Rhodospirillum rubrum* (16), the spectral changes due to carotenoids were observed in intact cells and not in disrupted cells.

**Inhibition of Carotenoid Formation by Growth in the Presence of Diphenylamine.** The presence of carotenoids interfered with the difference spectra of the cytochromes, therefore, diphenylamine (DPA) was added to the growth medium in an attempt to inhibit carotenoid formation (5, 7). At a final concentration of $1 \times 10^{-4}$M, growth was not inhibited, but the cells were nearly colorless, indicating an inhibition in carotenoid formation. The absolute spectrum of these cells is shown in figure 2 (line C); these cells are referred to as DPA cells. If a concentration of $3 \times 10^{-3}$M DPA was used, somewhat yellowish cells resulted. The absolute spectrum of such cells is shown in figure 2 (line B). The absorption at 420 $\mu m$ in figure 2.

**Fig. 4.** Time course of the changes in absorbancy of the difference spectrum of intact cells. The concentration of cell suspension was 12.0 mg/ml. At the point indicated by the small arrow, all the dissolved oxygen presumably has been taken up. The large arrows show the direction of reduction of respiratory components in the reference cuvette.

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The supernatant fraction from this centrifugation is referred to as fraction II. Fraction II was then centrifuged at 22,000 × g for 10 minutes. The sediment (fraction III) was suspended in 0.2 M, pH 7.0 phosphate buffer and the supernatant layer centrifuged at 150,000 × g for 30 minutes. The sediment from this centrifugation is termed fraction IV, and the supernatant fraction, V.

The difference spectra of each fraction were measured. Some of the results obtained with extracts of DPA-cells are shown in figure 6 and table II. Similar results were obtained with pigmented cells. Cytochrome content was calculated from the difference spectrum (hydrosulfite reduced minus aerobically oxidized) of each fraction using the method of Chance (2). Fraction I (disrupted cell suspension) had almost the same difference spectrum as that of whole cells. Cytochromes a, b and c and flavoprotein were present in fraction II (a mixture of intracellular particles and soluble components). In fraction III (large particles), the content of b-type hemoprotein was quite low. Fraction IV (small particles) had all the respiratory components, but the content of cytochrome c was somewhat low. The soluble fraction V had a large amount of cytochrome c and a small amount of b-type hemoprotein, but very little cytochrome a component. Large amounts of cytochrome c were present in the soluble fraction of cell free extracts regardless of the procedure used to disrupt the cells.

**NADH-Oxidation by Sub-Cellular Fractions.**

NADH-oxidation was measured by following the

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**Figure 5.** Difference spectrum of intact DPA cells (carotenoid-deficient). Solid line: hydrosulfite reduced; dotted line: endogenously reduced. Other conditions as indicated in figure 3.

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**Figure 6.** Difference spectra of fractions IV and V. The sample was reduced by hydrosulfite and the reference suspension oxidized by aeration. Protein concentrations of fractions IV (small particle) and V (supernatant) were 1.15 and 3.50 mg/ml, respectively.
optical density change at 340 μm and measuring O₂ uptake with the oxygen electrode. Both procedures gave equivalent values. The results obtained by the spectrophotometric procedure with extracts of DPA cells are shown in Table II. Similar results were obtained with pigmented cells. The NADH-oxidase activity of fraction I, II, III, and IV was strongly inhibited by cyanide and antimycin A. In fraction V, NADH-oxidizing activity was very weak and the oxidation was somewhat tolerant to both inhibitors. The activity was found to be highest in the small particle fraction IV, which was the fraction with the highest content of cytochrome α (Fig 6 and Table II). Although fraction V had a large amount of cytochrome c, NADH-oxidizing activity was very low, presumably because the fraction had a very low content of cytochrome α. The loss of NADH-oxidizing activity might be due to the solubilization of the cytochrome c component. Thus, the major portion of NADH oxidase activity in these extracts was associated with particles and inhibited by cyanide and antimycin A, even though respiration of whole cells is insensitive to these inhibitors.

Changes in the Difference Spectrum of Cell Free Extracts During Reduction by NADH. The difference spectrum of fraction II was also obtained using NADH as the reductant. The results with DPA cells are shown in Figure 7. Similar results were obtained with pigmented cells. At first the cytochrome c component appeared; after 3 minutes the cytochrome α and b components could be observed. Addition of hydrosulfite resulted in a further reduction of cytochromes b, c and flavoprotein.

Effect of Cyanide on the Growth of R. Glutinis. The effect of cyanide on the growth of the yeast was followed in order to determine if energy obtained from cyanide tolerant respiration could support growth. The pH of the medium (usually pH 6.3) was adjusted to 7.0 to minimize loss of cyanide. If young cells from a 2-day culture were used as the inoculum, growth was completely inhibited in the presence of 1 x 10⁻³M cyanide. Similar results were obtained when cells from a 4-day culture were used as the inoculum. Thus, although the respiration of the inoculum was not inhibited by 1 x 10⁻³M cyanide, growth was strongly inhibited by cyanide at this concentration.

Discussion

Difference spectra of DPA-cells (carotenoid-less) show that α, b and c-type cytochromes are present in R. glutinis. Based upon the pattern shown in figure 7, NADH can reduce cytochrome α, b and c and flavoprotein. NADH can be oxidized by sub-cellular particles and this oxidation is inhibited by relatively low concentrations of cyanide.

![Figure 7](attachment:image-url)

**Fig. 7.** Changes in the difference spectrum of cell free extracts (fraction II) of DPA cells. The base line was obtained, NADH added to the sample cuvette, and spectra taken at the times indicated. Extracts were reduced with hydrosulfite at the end of the experiment. Protein content 6.2 mg/ml.
and antimycin A (table II). These observations indicate that this yeast seems to have a cyanide-sensitive cytochrome-linked respiratory system such as is found in mammalian tissues and S. cerevisiae. Nevertheless, the respiration of whole cells is markedly resistant to respiratory chain inhibitors. Evidence for the possible mechanism of the cyanide insensitive pathway will be presented in another publication.

Since the growth of this yeast is inhibited by cyanide at a final concentration of $1 \times 10^{-3} \text{M}$, a concentration which has little or no inhibitory effect on $\text{O}_2$ uptake, we must conclude that a cyanide tolerant electron transport chain to oxygen does not play a significant role in the energy economy of this yeast.

The spectral changes in carotenoids observed upon aerations, are similar in some respects to the carotenoid changes produced by oxygenation or illumination in the photosynthetic bacteria Rhodopseudomonas spheroides and Rhodospirillum rubrum (11, 15, 16). These authors suggest that the carotenoids are not members of the respiratory electron transport chain even though aeration of intact cells causes changes in the carotenoid absorption spectra. The physiological significance of the carotenoid changes observed in R. glutinis, is not known.

Acknowledgments

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Literature Cited