

The Respiratory System of *Rhodotorula glutinis* II. Mechanism of Inhibitor Tolerant Respiration

Shoichi Matsunaka¹ and S. F. Conti^{2, 3, 4}

Department of Microbiology, Dartmouth Medical School, Hanover, New Hampshire

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Summary. The mechanism of inhibitor-tolerant respiration in *Rhodotorula glutinis* was studied. This inhibitor-tolerant respiratory pathway was not due to the presence of an excess of cytochrome *c* oxidase, nor to the operation of an inhibitor-resistant cytochrome *c* oxidase. Carotenoids do not appear to be involved in this respiratory chain pathway; data are also presented which show that the inhibitors penetrate into the cell. Although the initial rate of oxygen uptake by intact cells was not inhibited in the presence of cyanide or antimycin A, in the presence of these inhibitors the rate of oxygen uptake decreased significantly when the oxygen concentration fell below 100 μ M. This change in rate of oxygen uptake as a function of pO_2 , suggests that a respiratory chain with a low affinity for oxygen operates in the presence of inhibitors. The characteristics of this alternate pathway are described.

The respiratory system found in most cells is characteristically inhibited by agents such as cyanide or antimycin A. However, in the presence of an inhibitor, an insensitive respiratory system has been demonstrated in a variety of cytochrome-containing plant tissues (4). In general, 2 explanations have been invoked to account for oxygen uptake in the presence of respiratory chain inhibitors (e.g. cyanide): A) the operation of an alternate inhibitor insensitive pathway (1, 16); and B) the presence of an excess of a normal cyanide-sensitive cytochrome *c* oxidase (2). Cyanide tolerant respiration has also been demonstrated in tissues of the dormant Cecropia silkworm (14), and in the midgut of the mature silkworm (13). It was suggested in the latter case that an alternative pathway operated and that cytochrome b_5 could be acting as the inhibitor tolerant terminal oxidase.

Studies with cells of the bacterium *Achromobacter* adapted to growth in the presence of cyanide revealed the presence of much higher concentrations of the terminal oxidase (cytochrome a_2) in the adapted cells than in the sensitive cells (10, 11); the excess oxidase hypothesis therefore can account for the operation of this system. Recent observations of inhibitor tolerant respiration in the fungus

Myrothecium verrucaria suggest the presence of 2 cytochrome *c* oxidases, one of which is resistant to the action of some respiratory chain inhibitors (6).

Studies in our laboratory (9) have shown that O_2 uptake by intact cells of the yeast *Rhodotorula glutinis* is not affected by the presence of cyanide or antimycin A in concentrations which completely inhibit respiration of the yeast *Saccharomyces cerevisiae*. *R. glutinis* was shown to contain flavoprotein and cytochromes; results with cell free extracts suggested the presence of a cyanide and antimycin A sensitive, cytochrome-linked respiratory chain (9).

The purpose of the present study is to characterize and obtain information on the mechanism of inhibitor tolerant respiration in *R. glutinis*.

Materials and Methods

The materials and methods used in this study have been described (9). Cytochrome *c* oxidase activity of cell free extracts was measured by the method of Kuboyama and King (7), using a Clark oxygen electrode; Sigma horse heart cytochrome *c* (Type III) was used in the assay. Inorganic phosphate incorporation into intact cells was measured by incorporating inorganic ^{32}P (30 μ c) into the following reaction mixture; 10 ml of yeast cells (200 mg dry wt) in distilled water, 5 ml of 0.1 M phosphate buffer (pH 7.0), and 4 ml of 0.2 M glucose. The reaction mixture was brought to 30 ml with distilled water and incubated with aeration at 25°. After incubation in the presence or absence of inhibitor, 5 ml of the reaction mixture was centrifuged and the cells thoroughly washed with 0.1 M phosphate buffer. The cells were brought back to

¹ On leave from National Institute of Agricultural Sciences, Konosu, Saitama, Japan.

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³ Career Development Awardee, United States Public Health Service, IK 3-GM-8716.

⁴ Present Address: Department of Microbiology, University of Kentucky, Lexington, Kentucky.

volume, and duplicate 0.1 ml samples of the suspension were spread and dried on aluminum planchets. Incorporated radioactivity was determined using conventional procedures.

Results

The Relationships Between Carotenoid and Cyanide Insensitivity. Older cells of *R. glutinis* have a higher content of carotenoids, and their respiration is more tolerant to cyanide than younger cells (9). Difference spectra (endogenously reduced minus aerated) of intact cells revealed spectral changes in the carotenoid region (560, 520, 485 $m\mu$) which were not inhibited even in the presence of cyanide (fig 1). Since these observations suggested that carotenoids might be involved in cyanide tolerant respiration, we tested the cyanide sensitivity of normal cells, and carotenoid-deficient cells (prepared by growing cells in the presence of diphenylamine). As shown in table I, the absence of carotenoids did not result in cells which were sensitive to cyanide.

Carbon Monoxide Difference Spectrum. In order to determine if there were significant differences in the amount of terminal oxidase in young and old cultures, CO difference spectra were obtained. As shown in figure 2, the CO spectra of young and old cells are quite similar. The spectra suggest the presence of cytochrome a_3 (trough at 445 $m\mu$) and another CO-binding pigment (peak at 418 $m\mu$) in this yeast. These spectra suggest that the difference between young and old cultures in their sensitivity to cyanide is not correlated with large changes in the amount of CO-binding pigment. It is assumed that significant alteration in the amount of terminal oxidase would be reflected in the CO difference spectra.

Cytochrome c Oxidase and its Sensitivity to Cyanide. Since cyanide insensitive cytochrome oxidases have been observed in some microorganisms (6, 12), the sensitivity of the cytochrome *c* oxidase of *R. glutinis* to cyanide was tested. Cells were disrupted in a French press operated at 15,000 lb/sq in. The material was then centrifuged at $3,000 \times g$ for 10 minutes and the supernatant fraction was tested for cytochrome *c* oxidase activity.

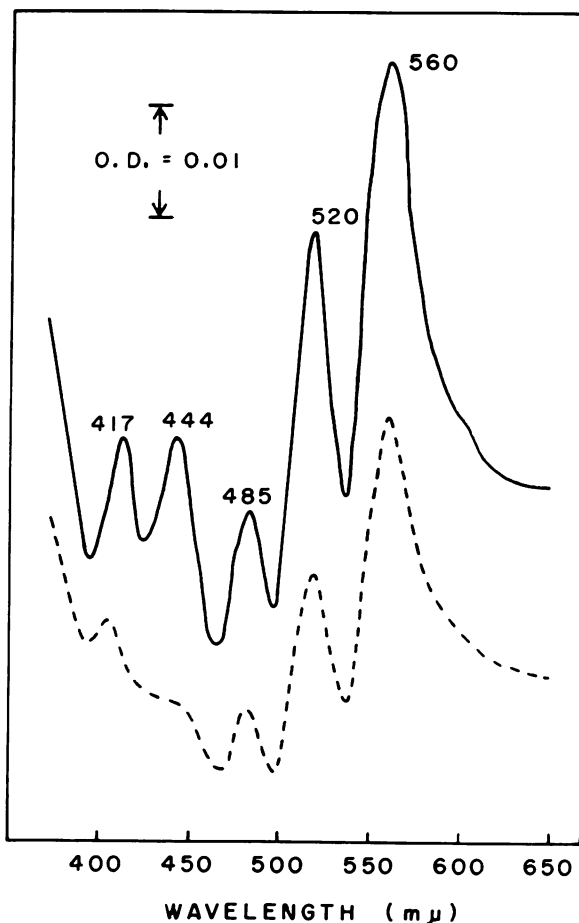


FIG. 1. Difference spectrum of pigmented cells in the presence of cyanide. Upper curve (—), without cyanide; lower curve (---), with 1×10^{-2} M cyanide. The reference cuvette was shaken vigorously for 2 minutes before scanning. Concentration of cells, 6.0 mg dry weight/ml.

This preparation had a specific activity of 0.22 to 0.40 $\mu\text{moles O}_2/\text{sec mg protein/ml}$. Cytochrome *c* oxidase activity was completely inhibited in the presence of 1×10^{-4} M cyanide as shown in figure 3.

The Effect of Oxygen Concentration in the Reaction Mixture. Polarographic measurements of

Table I. Cyanide Tolerant Respiration in Normal and Carotenoid Deficient Cells

Reaction mixture: yeast, 22 mg (dry wt); 0.1 M phosphate buffer, pH 7.0; 0.033 M glucose; total volume 3.0 ml. Measured polarographically at 23° using a Clark oxygen electrode.

Concentration of cyanide (M)	Normal cells		Carotenoid deficient cells	
	O ₂	inhibition* %	O ₂	inhibition* %
0	18.5	...	14.8	...
1×10^{-4}	21.3	- 15.1	15.9	- 7.6
1×10^{-3}	20.6	- 11.4	14.7	0.7
1×10^{-2}	17.1	7.6	13.0	12.2

* A negative value indicates a stimulation of respiration.

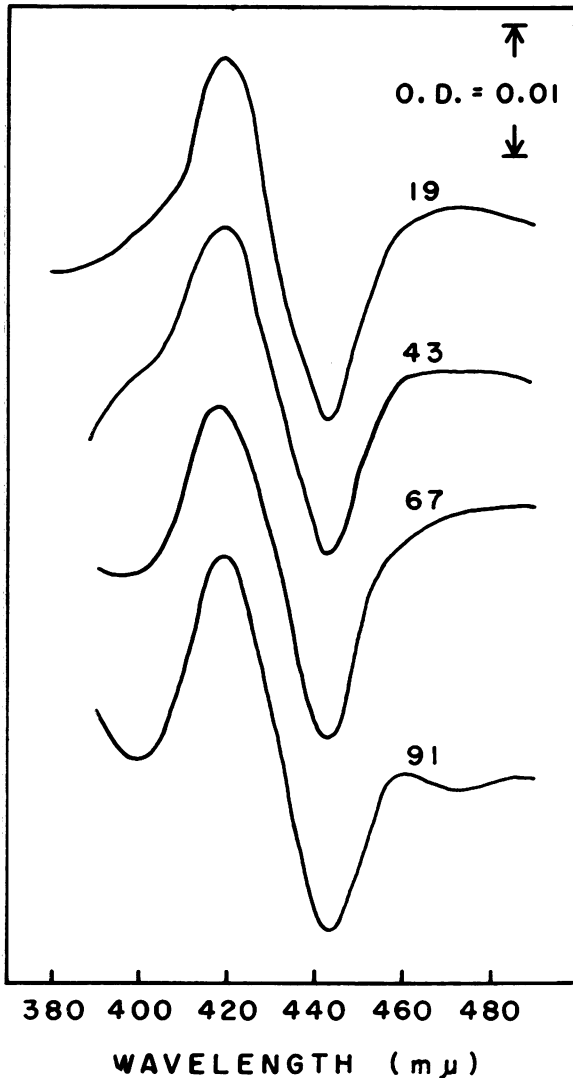


FIG. 2. Carbon monoxide difference spectrum of cells at difference ages. Hydrosulfite was added to both sample and reference cuvette, and carbon monoxide was bubbled through the sample cuvette for 3 minutes. Cell concentration, 7.0 mg dry weight/ml. Numbers indicate the age of the culture in hours.

oxygen uptake by intact cells showed that the velocity of oxygen uptake was constant down to very low levels of oxygen (fig 4a) indicating the operation of a respiratory system with a high affinity for oxygen. In the presence of cyanide, although the initial rate of O_2 uptake was not inhibited, the rate of oxygen uptake decreased significantly when the oxygen concentration fell below $100 \mu M$ (fig 4a). This change in rate as a function of pO_2 indicates the operation of a respiratory chain with a low affinity for oxygen. The respiratory velocities, calculated from the tangent of the trace curves, were plotted against concentration of oxygen in the reaction mixture (fig 4b).

As shown, the concentration of O_2 at which the rate of uptake is half-maximal is $4 \mu M$ in the absence of cyanide, and $23 \mu M$ in the presence of $1 \times 10^{-3} M$ cyanide. This phenomenon was observed

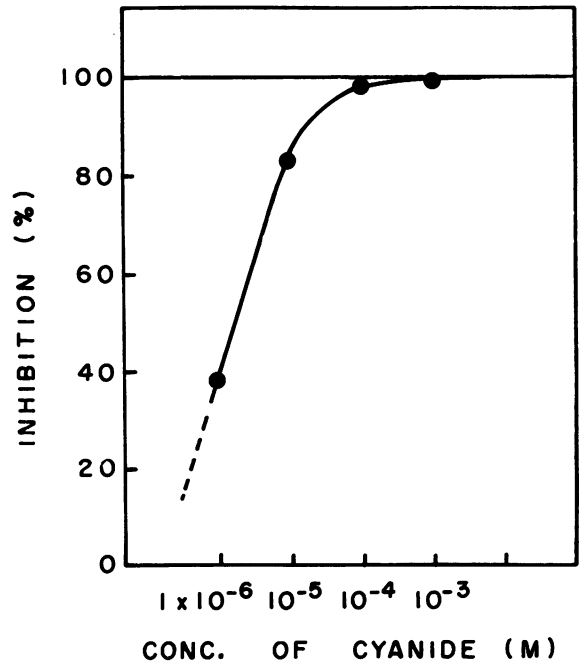


FIG. 3. Sensitivity of cytochrome *c* oxidase to cyanide. The reaction mixture contained in phosphate buffer (0.1 M, pH 7.0); ascorbate 15 mM, EDTA 0.6 mM, cytochrome *c* $20 \mu M$ and cell free extract (5.9 mg protein) in a total volume of 3 ml. Reaction temperature, 24° . Activity was calculated from the slope of the traces recorded polarographically using a Clark oxygen electrode.

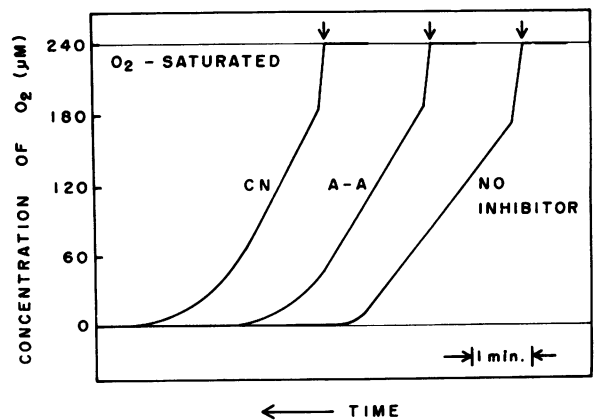


FIG. 4a. Trace of O_2 uptake by intact cells in the presence of inhibitor. Reaction mixture contained in phosphate buffer (pH 7.0, 0.1 M): glucose 3 mM, and yeast cells (16.0 mg dry weight) in a total volume of 3.0 ml. Reaction temperature, 24° . CN: 1.0 mM cyanide; A-A: 10 mg/l antimycin A. Yeast cells were added at the time shown by the small arrows.

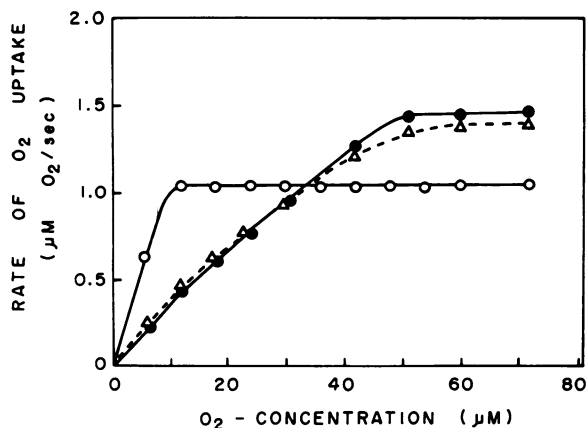


FIG. 4b. The effect of oxygen concentration on the velocity of oxygen uptake by intact cells. The velocity of oxygen uptake was calculated from the tangent of curves similar to those shown in figure 4a. (O—O) no inhibitor, (●—●) 1 mM cyanide, (Δ — Δ), 10 ppm antimycin A.

over a wide range of cyanide concentrations (1×10^{-5} – 1×10^{-2} M) and to the same extent indicating that this phenomenon did not result from a simple competition between O_2 and cyanide for cytochrome oxidase.

Aeration of the sample, after the cells had reached zero oxygen concentration, resulted in a O_2 uptake curve indistinguishable from the initial one suggesting that the decreased rate of O_2 uptake at low levels of oxygen concentration did not result from a gradual inhibition of the cells by cyanide. The effects of antimycin A (fig 4a, 4b), and CO were similar to those produced by cyanide. Rotenone as well as 2 heptyl- and 2 nonyl-4-hydroxyquinoline-N-oxide did not inhibit O_2 uptake and did not affect the rate of O_2 uptake at low levels of O_2 concentration.

Difference Spectrum in the Presence of Antimycin A. A normal difference spectrum, showing peaks of cytochrome *b*, *c* and *a₃* is evident when the reference cuvette is aerated (fig 5). However, when antimycin A is present in the sample cuvette and both cuvettes are shaken, only the peaks due to cytochrome *b* appear, (560, 530 and 430 $m\mu$) indicating that antimycin A blocks electron transport between cytochrome *b* and *c*. Under these conditions, a trough appears in the region of cytochrome *c*, suggesting that cytochrome *c* is more oxidized in the presence of antimycin A. When antimycin A was added to both cuvettes, and only the reference cuvette was shaken, the peaks of cytochromes *a₃* (603, 444 $m\mu$) and *c* (550, 520, 417 $m\mu$) were greater than that observed in the normal difference spectrum. These observations suggest that in *R. glutinis* antimycin A blocks electron transport between cytochrome *b* and *c*, just as it does in the respiratory chain of *S. cerevisiae*.

The Effect of Cyanide Upon the Incorporation of Inorganic Phosphate into the Cells. We have shown that respiration of this yeast was not inhibited by 1×10^{-3} M cyanide, whereas growth was inhibited almost completely (9). It was tentatively concluded that the electron transport chain involved in O_2 uptake in the presence of cyanide does not contribute to the production of ATP. In figure 6 the time course of ^{32}P incorporation into the cells is shown. In the absence of cyanide, P_i was incorporated into the cells throughout the experimental periods. In the presence of cyanide the incorporation was almost completely inhibited, although respiration was not affected.

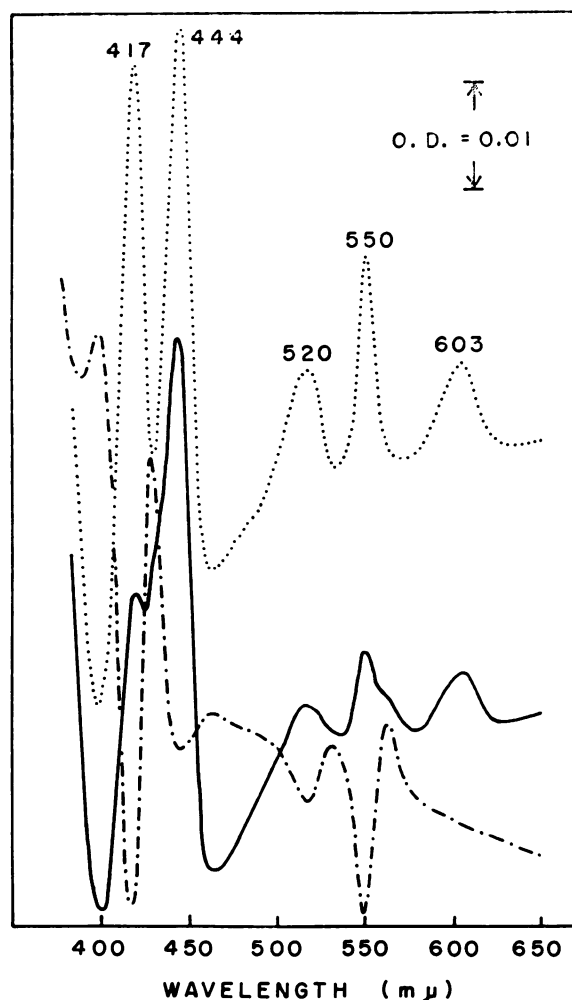


FIG. 5. Difference spectrum in the presence of antimycin A. Solid line (—), endogenously reduced minus aerated; dotted line (⋯), antimycin A (10 $\mu\text{g/ml}$) in both cuvettes, only the reference cuvette was aerated; dashed line (- - -), antimycin A (10 $\mu\text{g/ml}$) only in the sample cuvette, both cuvettes aerated. Concentration of cells, 16 mg dry weight/ml.

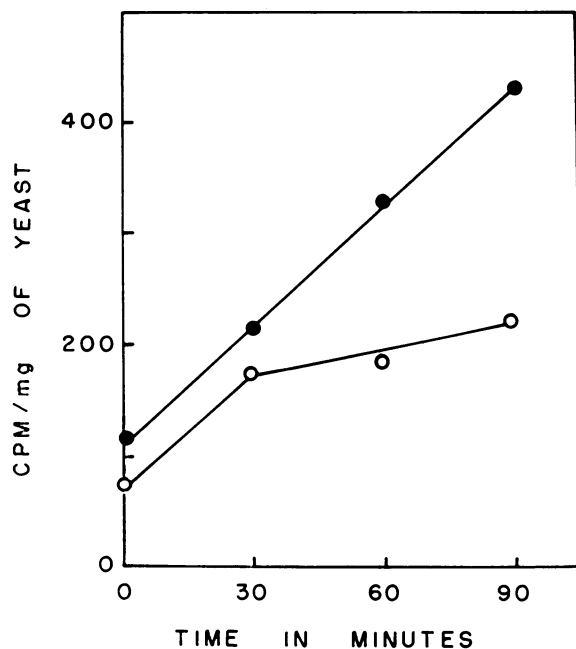


FIG. 6. The effect of cyanide on ^{32}P incorporation into cells. In absence (●—●), and in presence of 1 mM cyanide (○—○). Other experimental conditions are described in the text.

Discussion

Oxygen uptake by whole cells of the aerobic, carotenoid-containing yeast *R. glutinis* exhibits a high tolerance to cyanide and antimycin A (9). However, these cells possess a typical cytochrome system, and the NADH oxidase and cytochrome *c* oxidase activity of cell free extracts is very sensitive to cyanide. These results could be due to the impermeability of intact cells to cyanide or antimycin A. Preliminary studies indicated that over a wide range of pH, and despite extended incubation periods, cyanide and antimycin A did not inhibit O_2 uptake by intact cells. We also have shown (9) that concentrations of cyanide which did not inhibit respiration, completely inhibited growth. These observations together with the change in rate of O_2 uptake as a function of $p\text{O}_2$, and inhibition of ^{32}P incorporation into cells by cyanide, further substantiate the conclusion that cyanide can penetrate into the cells. The inhibition in whole cells by cyanide of absorbancy changes due to cytochromes further indicate penetration (fig 1).

Since oxygenation of cells induced significant absorbancy changes in the carotenoids of this yeast, even in the presence of cyanide (fig 1), carotenoid involvement in inhibitor tolerant respiration was considered as a possibility. The cyanide tolerant respiration of carotenoid-deficient cells, prepared by growth in the presence of diphenylamine, is so similar to that of normal cells, that we must con-

clude that the carotenoids do not play a significant role in the inhibitor tolerant respiratory chain of this yeast. The differential effect of cyanide on the absorbancy changes due to carotenoids and cytochrome further suggests that these pigments are not on the same electron transport chain.

The excess oxidase hypothesis maintains that in inhibitor tolerant systems, there is relative excess of a cyanide sensitive cytochrome oxidase over other respiratory components. As shown in *Achromobacter* (10,11), which contains an excess of cytochrome a_2 as its terminal oxidase, a considerable inhibition of the terminal oxidase does not result in a decrease in the electron flux. We wished to determine whether this hypothesis could explain the CN tolerance of *R. glutinis*. Since cyanide tolerance in *R. glutinis* is somewhat affected by the age of the cells, CO difference spectra of the cells at different ages were compared. As shown in figure 2 significant changes in the amount of CO-binding oxidase does not accompany increase or decrease in cyanide tolerance.

Since some strains of bacteria can oxidize CO to CO_2 (15), or assimilate it as a sole carbon source, the possibility existed that *R. glutinis* can detoxify cyanide or antimycin A. Since cyanide tolerant respiration can be demonstrated less than 10 seconds after mixing yeast cells with relatively high concentrations of cyanide, it is quite improbable that enzymatic detoxification of cyanide is involved in the tolerance mechanism. Furthermore, if detoxification of cyanide plays a significant role, the growth of the cells and the incorporation of P_i would not be inhibited by cyanide.

The existence of a cytochrome *c* oxidase in *R. glutinis* which is tolerant to cyanide, is highly improbable since cytochrome *c* oxidase activity of cell free extracts was completely inhibited by $1 \times 10^{-4}\text{M}$ cyanide. The fact that the respiration of whole cells of this yeast is insensitive to antimycin A as well as CN further negates this possibility. The situation in *R. glutinis* is therefore not analogous to those systems where an inhibitor tolerant cytochrome *c* oxidase has been demonstrated (12,16).

The main characteristics of the inhibitor tolerant respiratory chain of *R. glutinis* are as follows: The lowered rate of O_2 uptake occurs at low concentrations of oxygen ($<100 \mu\text{M}$), and only in the presence of cyanide. This decrease in the velocity of O_2 uptake occurs over a relatively wide concentration of cyanide, and the rate is independent of cyanide concentration. The decreased rate of O_2 uptake at low concentrations of O_2 can be brought back to the initial rate by aeration. CO and antimycin A also produce the same effects as cyanide; it was also noted that with some cell suspensions, the rate of O_2 uptake was somewhat stimulated by the presence of cyanide.

It seems reasonable to postulate the following mechanism to explain these observations. A normal cytochrome linked cyanide and antimycin A sensi-

tive pathway, similar to that found in *S. cerevisiae* and most other plant and animal tissue, is operative in *R. glutinis*. Characteristically, this pathway exhibits a high affinity for oxygen, and is readily demonstrable in cell free extracts. In the presence of inhibitors, this normal respiratory chain is blocked and an alternate pathway operates. The alternate pathway is characterized by a low affinity for oxygen. This pathway was demonstrable in whole cells but not in disrupted cells presumably because it is destroyed or diluted out when the cell is broken. The junction of this pathway with the normal respiratory chain must occur prior to the antimycin A site, since antimycin A induces the same effects as cyanide. Thus, the alternate pathway could involve flavoprotein and cytochrome *b*, but not cytochrome *c* or *a₃*. It has been pointed out (8) that tolerance to respiratory chain inhibitors may reflect either operation or a respiratory path not susceptible to inhibitors, or operation of an alternative compensatory respiratory path brought about by inhibition of the normal pathway by the inhibitor. In the case of *R. glutinis*, the inhibitors appear to exert their action on the normal respiratory chain, thereby resulting in the operation of a compensatory respiratory system. Our data suggest that the cyanide tolerant electron chain is non-phosphorylative and thus analogous to the situation in the mitochondria isolated from the skunk cabbage spadix and aged potato slices where cyanide inhibits oxidative phosphorylation but has no effect on respiration (3, 5).

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Literature Cited

1. BENDALL, D. S. AND R. HILL. 1965. Cytochrome components in the spadix of *Arum maculatum*. *New Phytologist* 55: 206-12.
2. CHANCE, B. AND D. P. HACKETT. 1959. The electron transfer system of skunk cabbage mitochondria. *Plant Physiol.* 24: 33-49.
3. HACKETT, D. P. AND D. W. HAAS. 1958. Oxidative phosphorylation and functional cytochromes in skunk cabbage mitochondria. *Plant Physiol.* 33: 27-32.
4. HACKETT, D. P. 1959. Respiratory mechanisms in higher plants. *Ann. Rev. Plant Physiol* 10: 113-46.
5. HACKETT, D. P., D. W. HAAS, S. K. GRIFFITHS, AND D. J. NIEDERPRUEM. 1960. Studies on development of cyanide resistant respiration in potato tuber slices. *Plant Physiol.* 35: 8-19.
6. KIDDER, G. W., III AND D. GODDARD. 1965. Studies on the inhibition resistant respiration of the fungus *Myrothecium verrucaria*. *Plant Physiol.* 40: 552-56.
7. KUBOYAMA, M. AND T. E. KING. 1964. Reconstitution of cytochrome oxidase from cytochrome *a* and heme *a*. *Biochim. Biophys. Acta* 92: 618-21.
8. LATIES, G. G. AND C. HOELLE. 1965. Malonate and cyanide insensitivity in relation to respiratory compensation in potato slices. *Plant Physiol.* 40: 757-64.
9. MATSUNAKA, S., S. MORITA, AND S. F. CONTI. 1966. The respiratory system of *Rhodotorula glutinis*, I. Inhibitor tolerance and cytochrome components. *Plant Physiol.* 41: 1364-69.
10. MIZUSHIMA, S. AND K. ARIMA. 1960. Mechanism of cyanide resistance in *Achromobacter*. II. Adaptive formation of cyanide resistant respiratory system in resting cells. *J. Biochem.* 47: 600-07.
11. MIZUSHIMA, S. AND K. ARIMA. 1960. Mechanism of cyanide resistance in *Achromobacter*. III. Nature of terminal electron transport system and its sensitivity to cyanide. *J. Biochem.* 47: 837-45.
12. OKA, T. AND K. ARIMA. 1965. Cyanide resistance in *Achromobacter*. II. Mechanism of cyanide resistance. *J. Bacteriol.* 90: 744-47.
13. PAPPENHEIMER, A. M. AND C. M. WILLIAMS. 1954. Cytochrome *b* and the dihydrocoenzyme I-oxidase system in the cecropia silkworm. *J. Biol. Chem.* 204: 915-29.
14. SCHNEIDERMAN, H. A. AND C. M. WILLIAMS. 1954. The physiology of insect diapause. VIII. Qualitative changes in the metabolism of the cecropia silkworm during diapause and development. *Biol. Bull.* 106: 210-29.
15. YAGI, T. 1959. Enzymic oxidation of carbon monoxide II. *J. Biochem.* 46: 949-55.
16. YOCUM, C. S. AND D. P. HACKETT. 1957. Participation of cytochromes in the respiration of the aroid spadix. *Plant Physiol.* 32: 186-91.