Terminal Oxidases of Chlorella pyrenoidosa$^{1,2}$

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ABSTRACT

In studies of the kinetics of oxygen uptake by glucose-stimulated Chlorella pyrenoidosa, two terminal oxidases could be distinguished. The cytochrome oxidase of Chlorella has a $K_m(O_2)$ of 2.1 ± 0.3 $\mu$M, while the second oxidase has a $K_m(O_2)$ of 6.7 ± 0.5 $\mu$M, and a maximum capacity about one-quarter of that of the cytochrome system. The identity of the second oxidase is unknown, but it is not inhibited by carbon monoxide, 1 $\mu$M cyanide, 0.1 $\mu$M thiocyanate, or 1 $\mu$M 8-hydroxyquinoline. In fresh cultures, the second oxidase accounts for at most 35% of the total oxygen uptake.

As early as 1919, Warburg (14) noted that the respiration of Chlorella was much more resistant to cyanide than that of many other types of cells. In 1926, Emerson (5) reported that the endogenous respiration of Chlorella was both cyanide and carbon monoxide insensitive, but that glucose respiration was partially inhibited by these two poisons, and the inhibition by carbon monoxide was light reversible. This behavior suggested that Chlorella has at least two separate oxidase systems, one of which is the widely found mitochondrial electron transport chain terminating in cytochrome oxidase. Syrett (12) has shown that a cyanide-insensitive oxidase may account for 30 to 60% of the normal oxygen uptake of C. vulgaris. The nature of the cyanide-insensitive oxidase is unknown, but Gibbs (6) has suggested that either a cyanide-resistant cytochrome (such as the autoxidizable cytochrome $b_4$) or a flavoprotein with a high oxygen affinity might be responsible. He pointed out, however, that the latter possibility is unlikely, because of the very high oxygen affinity ($K_m$ for $O_2 < 5$ mm Hg) reported by Tang and French (13).

Schonbaum et al. (11) and Bendall and Bonner (2) have reported on a cyanide-resistant respiration of mitochondria from higher plants. These authors reported a selective inhibition of the cyanide-resistant pathway by hydroxamic acids (11) and thiocyanate, $\alpha$,$\alpha'$-dipyridyl, and 8-hydroxyquinoline (2). Bendall and Bonner (2) concluded that the cyanide-insensitive oxidase is not a cytochrome, although the apparent $K_m(O_2)$ for this oxidase is only 0.5 $\mu$M (7). From a consideration of the class of substances found to be effective as inhibitors, namely, metal complexing agents, and the spectroscopic detection of the formation of ferrous complexes upon addition of the inhibitors, Bendall and Bonner (2) suggested that a nonheme iron protein may be responsible. There have been no reports of the effects of these agents on the respiration of Chlorella.

In this paper we report on a study of the oxidase systems of Chlorella pyrenoidosa: the number present, their oxygen affinities, inhibitor responses, and relative capacities.

MATERIALS AND METHODS

Chlorella pyrenoidosa Chick (Indiana Algal Culture Collection strain No. 252) was grown under sterile conditions in 250-ml Erlenmeyer flasks and bubbled continuously with a mixture of 2% CO$_2$ in air. The cultures were shaken at one cycle per second and illuminated with a mixture of “Cool-Ray” and “Gro-lux” fluorescent lights, the intensity at the surface of the cultures being about 10,000 lux. The culture medium is given elsewhere (10). For measurements, the cells were washed and resuspended in 5 mm phosphate buffer, pH 7.2, at a concentration of about 10$^6$ cells/ml.

Starved cells were obtained by placing the cells in fresh, autotrophic growth medium at least 15 hr before experiments were run. The cultures were aerated and shaken during this period but kept darkened. For measurements, the cells were resuspended in 5 mm phosphate buffer, pH 7.2.

When a stimulated respiration rate was required, cells were placed in buffer supplemented with 1% glucose, a concentration which should yield maximal stimulation (5). The cells were allowed to stand in this mixture for 2 hr to allow full development of the stimulation (4). Measurements were made in the same solution.

Measurements on the rate of O$_2$ uptake were made at 21.0 ± 0.5°C in a shallow, closed Plexiglas chamber described fully elsewhere (8). The signal from the Clark electrode was differentiated and automatically plotted as a function of the oxygen concentration (9), yielding the familiar reaction rate versus substrate concentration curve. The respiration rate in the presence of the various inhibitors was slow enough that time lags in the differentiating system were negligible.

RESULTS AND DISCUSSION

General Effects. Table I presents our results on the immediate effects of CN$^-$ and DNP$^2$ on the respiration rate of starved and fresh cells and cells treated with 1% glucose ("glucose respiration"). The responses are seen to be very different in these different conditions: starved cells show considerable en...

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$^2$ The work reported in this paper is part of the thesis submitted by D. F. Sargent in partial fulfillment of the requirements for the degree of Ph.D., University of Western Ontario, August 1971.

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$^5$ Abbreviation: DNP: 2,4-dinitrophenol.
Table I. Effects of Cyanide and Dinitrophenol on the Rate of Oxygen Uptake by Chlorella

The oxygen concentration was greater than 90% of the air saturated value in all cases. Where several repetitions were made, the standard error of the mean is indicated. Inhibitor concentrations are accurate to 10%.  

<table>
<thead>
<tr>
<th></th>
<th>Control Rates</th>
<th>Change( \times ) Caused by Cyanide</th>
<th>DNP (20 ( \mu )M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \mu ) moles/mg dry wt/hr</td>
<td>0.2 mm</td>
<td>2.0 mm</td>
</tr>
<tr>
<td>Starved cells</td>
<td>0.06</td>
<td>+55 ± 10</td>
<td>+12</td>
</tr>
<tr>
<td>Fresh cells</td>
<td>0.34</td>
<td>-35 ± 10</td>
<td>-65</td>
</tr>
<tr>
<td>Glucose respiration</td>
<td>0.43</td>
<td>-70 ± 10</td>
<td>-75</td>
</tr>
</tbody>
</table>

1 Typical values.  
2 Enhancement (\(+\)); inhibition (\(-\)).

hancement in the presence of cyanide, even at a concentration of 2 mM; fresh cells show some inhibition at 0.2 mM \( \text{CN}^- \), and a considerably greater inhibition at 2 mM \( \text{CN}^- \), and glucose respiration shows a large inhibition at 0.2 mM \( \text{CN}^- \), with only a slight difference between 0.2 and 2 mM \( \text{CN}^- \). The response to 20 \( \mu \)M DNP also shows a trend between starved cells and glucose respiration, going from a quite considerable stimulation in the former to no significant effect in the latter.

These results are readily understood on the basis of a differing utilization of, or flux through, separate oxidase systems. If there are two or more oxidase systems, one of which is more efficient than the other(s) at yielding energy for metabolic processes, then, if a relatively constant energy supply is to be maintained, the inhibition of the more efficient system could be expected to increase the flux through the other system(s), unless the latter was (were) already operating at full capacity. The cyanide-sensitive mitochondrial electron transport chain is highly efficient at converting the energy liberated during the dissimilation processes into a useful biological form (ATP), and one might thus expect the above model to apply. The results given in Table I bear this out completely: starved cells, in which one would not expect the oxidases to be the rate-limiting factor, show a considerable stimulation, indicating that alternate oxidase systems do have a significant excess capacity in this situation; glucose respiration, on the other hand, shows a very large inhibition in the presence of cyanide, indicating that the mitochondrial electron transport chain is carrying most of the respiratory load in this condition. The results with glucose respiration indicate both that the maximum capacity of the cytochrome chain is about three to four times that of the alternate oxidase system(s), and, as there is very little difference between the inhibition at 0.2 and 2 mM \( \text{CN}^- \), that the cytochrome chain is almost completely inhibited at 0.2 mM \( \text{CN}^- \). The observations that there is a significant difference between the effects of 0.2 and 2 mM \( \text{CN}^- \) with both starved and fresh cells show that even the residual activity of the cytochrome chain at 0.2 mM \( \text{CN}^- \) makes up a considerable portion of the total respiratory uptake, indicating that in the absence of \( \text{CN}^- \) most of the oxygen uptake is probably mediated by cytochrome oxidase.

The findings with DNP (Table I) are consistent with the preceding discussion: the lack of significant change with glucose respiration could mean either that the cytochrome chain is operating at full capacity, or that the supply of substrate to the electron transport chain is rate limiting.

**Kinetic Studies.** To gain more detailed knowledge about the oxidase systems present in *C. pyrenoidosa*, and particularly to determine their kinetic parameters, we studied the dependence of respiration rate on oxygen concentration, using both cyanide and carbon monoxide to help distinguish the contributions of the cytochrome chain.

The determination of \( K_m \) values is not feasible with starved or fresh cells, as the oxidases are not rate limiting, except at extremely low oxygen concentration or very high inhibitor concentrations. The addition of glucose, however, will bring the flux through the oxidases to a level much nearer their maximum capacity, allowing one to observe the effects of inhibitors at both lower inhibitor concentrations and higher oxygen concentrations. As an illustration of the difference in respiratory kinetics found in the presence of glucose, we note that the apparent \( K_m \) for \( \text{O}_2 \) of fresh cells, *i.e.*, the oxygen concentration at which the respiration rate has dropped to half its maximum rate, is about 0.55 \( \mu \)M, whereas the corresponding value for glucose respiration is more than three times this (see Table II).

To confirm our previous conclusions about the extent of inhibition of the cytochrome chain by various cyanide concentrations, we looked at the kinetics of \( \text{O}_2 \) uptake in the presence of 0.06, 0.2, and 1 mM cyanide. Examples are presented in Figure 1, and the numerical results are summarized in Table II. At low cyanide concentrations the measured points do not yield a straight line, indicating contributions from at least two enzyme systems. At 1 mM \( \text{CN}^- \), however, the curve indicated

![Fig. 1. Kinetics of oxygen uptake by glucose-stimulated *C. pyrenoidosa* in the presence of cyanide. The triangles are calculated from the experimental values, while the circles have been corrected for the uptake found with 1 mM \( \text{CN}^- \) (curve c). (Where uncertainties are not indicated, they are comparable to the size of the symbol.) The apparent inconsistency of the two points with smallest V, S ratios in graph a) is an indication that the terminal oxidases are not rate limiting above the corresponding oxygen concentration (about 10 \( \mu \)M) in nonpoisoned cells.](https://www.plantphysiol.org/)

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Table II. Summary of the Enzyme Parameters Found with and without Cyanide

<table>
<thead>
<tr>
<th>Cyanide Conc (mM)</th>
<th>Oxidase System I</th>
<th>Oxidase System II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vmax (µM/min)</td>
<td>Km (µM)</td>
</tr>
<tr>
<td>0</td>
<td>23.5 ± 0.5</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>0.06</td>
<td>1.9 ± 0.3</td>
<td>9 ± 4</td>
</tr>
<tr>
<td>0.2</td>
<td>≈2</td>
<td>≈35</td>
</tr>
<tr>
<td>1.0</td>
<td>6.1 ± 0.2</td>
<td>6.7 ± 0.5</td>
</tr>
</tbody>
</table>

1 Presumably cytochrome oxidase.
² Constants assumed in deriving the corrected curves.

by the measured values cannot be distinguished from a straight line, implying either that only one system is making a significant contribution, or that the cyanide-sensitive and cyanide-insensitive systems have very similar Km values at 1 mM CN⁻: that the former is the case will be evident shortly. Assuming this result for the moment, one then concludes that the reaction represented in Figure 1c represents the contribution of the cyanide-insensitive oxidase(s). On the assumption that this contribution will be present in all trials, we corrected the other curves for this component (e.g., graph 1b). The linearity of the corrected values substantiated the procedure, and showed that the complex curves can be explained by the participation of cyanide-sensitive and cyanide-insensitive components. The ratio of the maximum velocities of the uninhibited cyanide-sensitive system to the cyanide-insensitive system is almost exactly 4:1. We cannot exclude the possibility that the cyanide-insensitive system contains several independent oxidases, but as the kinetics of the respiration provide no justification for postulating more than a single cyanide-insensitive oxidase, we will henceforth assume that only one, having a Km of 6.7 ± 0.5 µM, is involved.

Several points about the cyanide trials remain to be considered. First, with no CN⁻ present, although the uncertainty of the experimental points allows them to be fitted by a straight line, we feel that the results of the other trials justify applying the correction procedure to this case as well, and we take the parameters derived from the corrected curve to be the proper values. Second, from the trend of the Vmax and Km values of “oxidase system I,” the cyanide-sensitive system (Table II), it is obvious that at 1 mM CN⁻ this system will have a large Km and small Vmax, and will therefore not be making a significant contribution to the total respiratory rate, justifying the assumption made earlier that this was the case. Third, from the results quoted for oxidase system I in Table II, we see that the cyanide-sensitive pathway is more than 90% inhibited at 0.2 mM CN⁻, confirming the suggestion of the previous section that this system is almost completely inhibited at 0.2 mM CN⁻.

Finally, we note that the results shown for oxidase system I are consistent with reports of other authors on the cytochrome chain of microorganisms. Winzler (15), for example, working with yeast, found that cyanide both increased the Km for oxygen and decreased the Vmax. As the mechanism of cyanide inhibition is complex, possibly involving three different sites in the cytochrome chain (15), we do not attempt a detailed analysis of our data on the partially inhibited cytochrome system. The Km(O2) for the inhibited system, 2.1 µM, is similar to the value of 1 µM for yeast (15), but much higher than the value of 0.1 µM usually reported for the cytochrome oxidase of higher plants (3).

While there is no doubt that the cytochrome chain is part of the “cyanide-sensitive system”, other cyanide-sensitive oxidases, such as the ascorbic acid and phenol oxidases of many plants (1) do exist and might be contributing to the cyanide-sensitive reactions. Of these three oxidase systems, both cytochrome oxidase and phenol oxidase are competitively inhibited by CO, the inhibition of ascorbic acid oxidase by CO being very slight (1). Only with cytochrome oxidase is the inhibition reversed by light. Emerson (5) showed that glucose respiration was partially sensitive to CO, and that this inhibition was light-reversible. These results indicate that any contribution by phenol oxidase must be minor, but do not show anything about a contribution by ascorbic acid oxidase.

This latter question should be settled by comparing the inhibitions by CN⁻ and CO: as ascorbic acid oxidase is sensitive to the first, but not the second of these agents, a different correction factor would be required to isolate the CO-sensitive respiration than the CN⁻-sensitive respiration if ascorbic acid oxidase were playing a significant role.

A test of this is shown in Figure 2, where the results of two trials at very different CO concentrations are shown, with and without a correction for the cyanide-resistant respiration in the
manner described previously. If there were a significant contribution from a cyanide-sensitive, CO-insensitive oxidase, such as ascorbic acid oxidase, then the "corrected" values would not indicate a linear relationship. That such a relationship is, in fact, found shows that CN⁻ and CO are inhibiting the same components, and there is thus little or no contribution from ascorbic acid oxidase. The third graph in Figure 2 gives the relationship between the corrected Km values and the CO concentration. The linearity of the curve confirms the competitive action of CO, as would be expected for cytochrome oxidase.

As a further confirmation that phenol and ascorbic acid oxidases are not making a significant contribution to the over-all respiration, we note that the relatively large values of the Km values for oxygen of these oxidases (1) should make any contribution readily detectable at higher oxygen concentrations, but no such effect was observed.

Further Inhibitor Trials. Bendall and Bonner (2) reported that the cyanide-resistant respiration of plant mitochondria is selectively inhibited by thiocyanate and 8-hydroxyquinoline. We have tested these agents on glucose respiration of Chlorella and find no inhibition of respiration in the presence of either 0.1 mM thiocyanate or 1 mM 8-hydroxyquinoline (an enhancement is found with starved or fresh cells in the presence of 8-hydroxyquinoline) and no synergistic effect of these agents with cyanide, as has been reported for higher plants (2). That there is some enhancement by starved or fresh cells indicates that 8-hydroxyquinoline can probably enter the cells, and thus the lack of effect on glucose respiration shows that the cyanide-resistant respiration in Chlorella is probably mediated by a different oxidase than the cyanide-resistant respiration of higher plants.

We cannot say if the second oxidase branches from the cytochrome chain or is completely separate. Antimycin A (up to 3 μg/ml) and amytal (up to 10 mM) had very little effect on the respiration of intact cells. We assume this is because of a poor penetration of the inhibitors into the cells, which limits their application in these circumstances.

SUMMARY AND CONCLUSIONS

Our results show that the observed characteristics of the uptake of oxygen by Chlorella can be explained on the basis of two terminal oxidases. One of these, presumably cytochrome oxidase, has a $K_m$ of 2.1 ± 0.3 μM and accounts for the bulk of the normal respiratory oxygen uptake. The exact contribution to the respiration of fresh cells can not be judged, but from Table I we see that at least 65% of the uptake occurs through this oxidase. The actual contribution is undoubtedly higher, as when the cytochrome chain is inhibited the flux through the alternate oxidase could be expected to increase.

The identity of the cyanide-insensitive pathway remains a mystery. We have found it to have a $K_m$ for oxygen of 6.7 ± 0.5 μM and a maximum capacity of about 25% of that of the cytochrome chain. It is not inhibited by the metal-complexing agents thiocyanate and 8-hydroxyquinoline, showing that it is probably different from the cyanide-resistant oxidase of plant mitochondria. The $K_m$ for O₂ (6.7 μM) is also very different from the value of 0.5 μM reported for mung bean mitochondria (7). Our value for the $K_m$ is consistent with that reported for C. pyrenoidosa by Tang and French (13), who found that it must be less than 5 mm Hg (11 μM).

LITERATURE CITED