Short Communication

Fermentative Metabolism of *Chlamydomonas reinhardtii*¹

II. ROLE OF PLASTOQUINONE

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ABSTRACT

Evidence is presented to substantiate a chloroplastic respiratory pathway in the green alga, *Chlamydomonas reinhardtii*, whereby reducing equivalents generated during the degradation of starch enter the thylakoidal chain at the plastoquinone site catalyzed by NADH-plastoquinone reductase. In this formulation, the reduced plastoquinone is oxidized either by the photoevolution (photosystem I) of H₂ under anaerobic conditions or by O₂ during dark respiration.

In the preceding paper (7), we reported on the effect of light on the fermentative mode of starch breakdown in a strain of *Chlamydomonas reinhardtii* adapted to a hydrogen metabolism. When compared to cells kept in the dark, starch breakdown in the illuminated cells was decreased and the fermentative pattern was modified. Inasmuch as the rate of starch breakdown was restored on addition of the uncoupler, FCCP³, the light-induced inhibition was attributed to the role of the energy charge in regulating the glycolytic flux, in a way similar to that proposed to account for the Pasteur effect in this alga (15). The major modification of the fermentative pattern which was not reversed by FCCP or DCMU was a drop in ethanol yield relative to starch consumed. While, in the dark, ethanol formation accounted for the oxidation of the bulk of the reduced pyridine nucleotides generated in the glycolytic pathway, ethanol production in the light was a minor sink for reducing equivalents. To account for this difference, we (7) proposed that, in illuminated cells, most of the reducing equivalents formed during the oxidation of glyceraldehyde 3-P to glyceraldehyde 3-P were diverted to the thylakoidal chain at the plastoquinone site (1) with this transfer being catalyzed by NADH-plastoquinone oxido-reductase (9), thus halting the reduction of acetyl-CoA to acetaldehyde and subsequently to ethanol. PSI would catalyze the reoxidation of the reduced plastoquinone and the electrons would bleed off as H₂.

Making use of DBMIB, a plastoquinone antagonist (3) which is known to prevent not only reduced plastoquinone reoxidation but also noncyclic and cyclic photosynthetic electron transport, we report in this brief communication data substantiating the central role of plastoquinone as a component of a thylakoidal respiratory chain which is involved in starch degradation both in the light under anaerobiosis and during dark aerobic respiration. A preliminary report of this research has been published (8).

MATERIALS AND METHODS

Algae and Growth Conditions. *Chlamydomonas reinhardtii* (Dangeard), mutant strain F-60 lacking ribulose 5-P kinase, obtained from R. K. Togasaki, Indiana University, was grown in batch cultures on an acetate-supplemented medium (7) and harvested at the end of the exponential phase. *Chlamydomonas reinhardtii* 11-32/90 (strain 90), also called 11-32/b, is a wild type strain, from the Pflanzenphysiologisches Institut der Universität, Göttingen, F.R.G. It was grown photoautotrophically in synchronous cultures (13) and was harvested at the end of the light phase. At harvest, the cells were large and motile. Cell density was 1 to 2·10⁹ cells/ml; Chl content was 30 to 80 μg/10⁷ cells, and starch content was 6 to 12 μmol C/10⁷ cells.

Experimental and Analytical Procedures. Many of the experimental procedures have been described in the earlier paper (7) and only significant modifications are given here. Instead of Warburg flasks, 38- or 160-ml bottles were used for incubating the cells in atmospheres of varying partial pressures of O₂. Unless mentioned otherwise, cells were incubated for 30 min in the dark in N₂ (less than 5 μL O₂/L) before the light (100 w/m²) was turned on or O₂ was introduced. When O₂ was included in the atmosphere of the bottle, pure O₂, taken from 1 atm reservoir, was injected with a syringe through the serum cap. To maintain constant pressure, the same volume as injected was retrieved. Analytical methods for starch and ethanol are given in Reference 7. DBMIB, a gift from A. Trebst, University of Bochum, F.R.G., and G. Hind, Brookhaven National Laboratory, was dissolved in 100% methanol. The final concentration of methanol did not exceed 0.1% (v/v).

RESULTS AND DISCUSSION

Plastoquinone and Anaerobiosis. The addition of 10 μM DBMIB to darkened cells breaking down starch under N₂ has no effect on the molar yield of ethanol and H₂ (Table 1). In sharp contrast, ethanol production by the illuminated cells is restored by the quinone antagonist up to a value higher than that observed in the control (cells kept in the dark). With respect to ethanol yield, DCMU does not mimic DBMIB, an indication that this specific alteration of the light-induced fermentative pattern is not only mediated by a block in the thylakoidal electron transport chain, but at a specific site, namely, plastoquinone. Concomitant with an increase in ethanol formation, we observe a decrease in H₂ photoproduction to a level even less than that recorded for

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³ Abbreviations: FCCP, carbonyl cyanide-p-trifluoromethoxyphenyl hydrazone; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone.
Table 1. Effects of DBMIB and DCMU on the Fermentative Pattern of Starch Breakdown of C. reinhardtii F-60

Cells were adapted for 30 min in N\textsubscript{2} and incubated for 3.5 h in N\textsubscript{2} in the dark or in the light (100 w/m\textsuperscript{2}). DCMU and DBMIB (10 \textmu M each) were added before adaptation. Hydrogen evolution was determined by manometry and ethanol by enzymic assay. The data represent two separate experiments.

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>Products Formed/ Starch Glucose Consumed</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Ethanol</td>
</tr>
<tr>
<td>Dark</td>
<td>0.83</td>
</tr>
<tr>
<td>Dark + DBMIB</td>
<td>0.31</td>
</tr>
<tr>
<td>Light</td>
<td>1.12</td>
</tr>
<tr>
<td>Light + DBMIB</td>
<td>0.35</td>
</tr>
<tr>
<td>Light + DCMU</td>
<td>0.64</td>
</tr>
<tr>
<td>+ DCMU</td>
<td></td>
</tr>
</tbody>
</table>

![Graph](image)

**Fig. 1.** Effect of DBMIB and DCMU on anaerobic starch breakdown by C. reinhardtii 90 and F-60 in dark and light. Cells were adapted in N\textsubscript{2} for 30 min and incubated for 3.5 h in dark or light (100 w/m\textsuperscript{2}). DCMU and DBMIB were added before adaptation. (O, •), C. reinhardtii 90, light and dark, respectively. (□, △), C. reinhardtii F-60, light and dark, respectively. (Δ), C. reinhardtii F-60, light and 10 \textmu M DCMU.

DCMU.

The results obtained with DMCU and DBMIB are consistent with the proposal presented earlier (7) that the reducing equivalents generated in the glycolytic pathway are converted to H\textsubscript{2} by a light-driven electron flow system involving plastoquinone as a component and are not available for the reductive phase of the fermentative pattern. That DBMIB inhibits H\textsubscript{2} photoproduction more than DCMU is not unexpected inasmuch as DCMU eliminates only water as a source for H\textsubscript{2} while the quinone analog blocks H\textsubscript{2} evolution both from water and from starch. The residual H\textsubscript{2} evolved in the presence of the two inhibitors approaches that found in the darkened cells in amount and is most likely attributable to the sequence NAD(H)\textsuperscript{+}→FAD→H\textsubscript{2} demonstrated in a reconstituted spinach chloroplast preparation fortified with algal hydrogenase (14).

Concomitant with the restoration of ethanol production, DBMIB at a concentration of 10 \textmu M relieves completely the light-driven inhibition of starch breakdown without affecting the dark rate (Fig. 1). DCMU does not remove the block induced by the illumination. Similar data are found for both the mutant (F-60) and the wild type (90) strains. Inhibition of starch loss by light may be the resultant of many factors; but, clearly, a rise in the energy charge is an attractive one to account for this effect. An increase in the energy charge has been documented in Chlorella under comparable conditions (12). The increase in observed glycolysis on addition of DBMIB (Fig. 1) and FCCP (Fig. 3 in Ref. 7) to illuminated cells may be due to their effect on the energy charge inasmuch as both compounds block the photophosphorylation process (3). When a rise in the energy charge is slow or absent, ample Pi is available for rapid starch breakdown. In contrast, since DCMU does not block light-induced cyclic electron flow, its effect on the energy charge is less significant, and starch breakdown is consequently less affected.

**Plastoquinone and Aerobiosis.** We have presented data in Table 1 indicating that reducing equivalents generated in the light during the glycolytic dissimilation of starch can be removed as H\textsubscript{2} gas resulting in a decreased yield of ethanol when compared to fermentation in the dark. On the basis of the results obtained with DBMIB, a pivotal role was assigned to the chloroplastic plastoquinone pool to account for the altered fermentation pattern induced by light.

While the machinery for fermentation has been maintained in Chlamydomonas, the organism, typical of the green algae, is basically an obligate aerobe. It became of interest, therefore, to determine whether the pathway linking NADH with the plastoquinone pool is involved in starch degradation under the conditions of darkness and O\textsubscript{2}, i.e. dark respiration. Similar to light, aerobiosis would cause the elimination of ethanol formation inasmuch as O\textsubscript{2} rather than acetyl CoA would serve as the sink for electrons set free during glycolysis. Measurement of ethanol is a legitimate indicator of fermentative electron flow in F-60 since the organism does not reassociate alcohol (data not shown). On the other hand, H\textsubscript{2} evolution cannot be used as a marker since the enzyme hydrogenase is inactivated completely in air and partially by the presence of O\textsubscript{2}. At air levels of O\textsubscript{2}, ethanol formation is eliminated by the rates of 2% O\textsubscript{2} uptake and starch loss are not affected by 10 \textmu M DBMIB (data not shown), a concentration of inhibitor reported to be ineffective with respect to mitochondrial electron flow (16).

We conclude from these results that the chloroplastic plastoquinone pathway if it is involved in starch degradation is clearly not a rate limiting factor during dark respiration at 21% O\textsubscript{2}. We then explored the effect of DBMIB and O\textsubscript{2} concentrations between 0 and 5% on starch loss coupled to ethanol formation to determine whether the chloroplastic pathway is primarily a 'relief' bioenergetic process (4) and of limited physiological importance since the key enzyme, NADH plastoquinone oxido-reductase, has not been detected in higher plant chloroplasts (1, 9). Additionally, this approach would furnish further insight into the regulation of starch breakdown by O\textsubscript{2} (Pasteur effect) (15) and provide for the first time a measurement of the extinction point (the lowest concentration of O\textsubscript{2} which eliminates aerobic fermentation as measured by ethanol formation) in a green alga. Starch disappearance in the dark when plotted against the partial pressure of O\textsubscript{2} from 0 to 5% in the atmosphere above the algal suspension is inhibited maximally at 2% O\textsubscript{2} (Fig. 2). It would appear that, at this critical tension of 2% O\textsubscript{2}, fermentation as judged by ethanol appearance is completely suppressed and the algal cells prefer O\textsubscript{2} rather than the fermentative dismutation mode as a means of regenerating oxidized redox carriers. The strongest accelerative effect of DBMIB on starch degradation is observed at that O\textsubscript{2} tension (extinction point) at which breakdown is minimal compared to the control (N\textsubscript{2}). The plastoquinone analog extends the extinction point from 2% up to at least 5% O\textsubscript{2}. Finally, an extinction point of roughly 2% is consistent with that reported for higher plants (17). The observed increase
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The oxidation of glyceraldehyde-3-P to glyceraldehyde-3-P. The additional ATP formed by the thylakoidal pathway coupled to low levels of O₂ would play a key role in accounting for the oxygen-reaction coupled to dark CO₂ reduction (6).

Finally, plastoquinone-mediated respiration is apparently not the sole mechanism available to the Chlamydomonas chloroplast for the turnover of NAD(P)H since DBMIB loses its effect beyond 5% O₂. We are aware that DBMIB can act as a bridge molecule with O₂ but it appears not to catalyze a Mehler-type reaction at the concentrations (11) used in this study and, indeed, less ethanol would be produced. It is, therefore, not improbable that additional chloroplast electron flow mechanisms involving Fd (14) or glutathione reductase (10) are concomitant with, or may replace completely, the plastoquinone-mediated pathway at air levels of O₂.

Concluding Remarks. The chloroplast respiratory pathways provide the cell with the option of disposing of electrons formed during the degradation of starch to glyceraldehyde-3-P by transferring them to various terminal electron acceptors (13) including O₂, nitrite, protons, and sulfate. An important byproduct is ATP required in the phosphorylation of chloroplastic fructose-6-P. As a result of the recent availability of an intact, functional chloroplast from C. reinhardtii (13), it should be possible to sort out the pathways coupling carbohydrate degradation and the many ultimate electron acceptors, most of which require Fd as an intermediate redox carrier.

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Fig. 2. Effect of DBMIB on starch breakdown and ethanol production by C. reinhardtii F-60 at various partial pressures of O₂ in the dark. The cells were adapted for 30 min in N₂, various amounts of O₂ were introduced, and the cells were incubated for 3.5 h in the dark. DBMIB (10 μM) was added before adaptation. The data are the mean and SD of duplicates. A, Starch breakdown; ( ), control; ( ), 10 μM DBMIB; ( ), relative increase. This value is calculated as:

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\text{starch consumed with DBMIB} - \text{starch consumed in control} \\
\text{starch consumed in control}
\]

B, Ethanol formation; ( ), control; ( ), 10 μM DBMIB.

in the rate of starch breakdown under the conditions of 10 μM DBMIB and 2% O₂ can be partly explained by a continuation of the fermentative mode.

From our results, it is clear that C. reinhardtii possesses a plastoquinone-mediated respiratory pathway coupling the degradation of starch to O₂. The data are not inconsistent with the chloroplastic respiratory scheme proposed by Bennoun for Chlorella (2), and with an O₂-induced reoxidation of the photosynthetic electron transport chain in algal cells after anaerobiosis (5). A Pasteur effect (Fig. 2) at the low O₂ partial pressures may be indicative of the formation of an electrochemical gradient across the thylakoidal membrane which would increase the energy yield per molecule of glucose in addition to the substrate phosphorylations generated during the oxidation of glyceralde-