Effect of Light on Respiration and
Development of Photosynthetic Cells

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I. Hydrogen Metabolism

A. Chloroplast stability using the reconstituted preparation.

There have been many recent reports on H₂ photoevolution utilizing reconstituted systems consisting of chloroplast particles from higher plants, ferredoxin, and hydrogen functioning under essentially anaerobic conditions. Bennemann et al (1) have reported a system that produced H₂ by the biophotolysis of water but the system was unstable, losing about 50% of activity after 15 min, apparently due to the presence of evolved O₂. This inhibition could not be accounted for by O₂ inactivation of hydrogenase activity. Improvements were noted in a report by Rao et al (2) with respect to both rates and stability. Maximum lifetime for their system was six and one-half hr at rates of 10 $\mu\text{mol H}_2/\text{mg Chl}\cdot\text{hr}$. Further improvements in rate and stability were observed by Fry et al (3). Their photosystem (PS) II-dependent system supported H₂ photoevolution at rates of 15 to 20 $\mu\text{mol}/\text{mg Chl}\cdot\text{hr}$ for up to 2 hr with much reduced rates up to 20 to 24 hr. Employment of O₂ traps, while increasing the efficiency and rate of H₂ photoproduction, did not extend the lifetime of the system. Since hydrogenase remained active, loss of system activity was attributed to inactivation of PS II and a loss of the coupling between low potential electrons and hydrogenase. We have investigated the effects of anaerobic conditions on the stability of higher plant and algal chloroplast particles.

Preparations of algal and higher plant particles were subjected to

air (21% O₂) of N₂. The results are summarized in Table 1. Algal particles demonstrated a sensitivity to imposed anaerobicity. In wild type (Wt) Chlamydomonas, the rate of ferricyanide reduction was reduced over 90% under N₂ but recovered 70% of the original rate when reexposed to air. In control experiments, particles from Chlamydomonas Wt lost from 12-15% of original ferricyanide reducing capacity per hr when not exposed to N₂. When Euglena or F60 were examined for anaerobic sensitivity, inhibition was noted, but only 50 to 55% of the capacity of aerobic ferricyanide reduction was lost. Reexposure to air did not result in a recovery of the original activity. Loss of activity to aerobic ageing was only 3 to 5%.

Spinach and peas demonstrated no significant inhibition by anaerobic conditions (Table 1). Spinach particles were quite unstable; activity declined 15 to 20% per hr even in the absence of gas exchanges. Pea particles were relatively stable, losing 3 to 5% of activity per hr due to ageing.

Algal particles, sensitive to anaerobic conditions, were found to be uncoupled by preparation as shown by the addition of 1mM NH₄Cl or 1μM F₃CCP having no stimulatory effect on either O₂ evolution or ferricyanide reduction (data not shown). On the other hand, the rate of ferricyanide reduction by pea particles was enhanced by these uncouplers (Table 1) and inhibited 50 to 60% by anaerobiosis following uncoupling. The uncoupled higher plant particles not only did not recover significant activity upon reexposure to air, but lost much of their remaining ferricyanide reducing capacity.

The effect of anaerobicity on the ability of chloroplast particles to reduce ferricyanide is apparently related to the coupling of electron

transport to the photophosphorylation mechanism. Algal systems, uncoupled by sonic oscillation, were sensitive to inhibition by the imposition of anaerobic conditions by 50%, but retained that level of activity even when reexposed to air. Chlamydomonas Wt, although uncoupled in preparation, appeared to be more sensitive to anaerobicity with respect to ferricyanide reduction. On the other hand, this particle recovered most of it's capacity upon reexposure to air. The lack of recovery in F60 and Euglena may be an indication of mechanical breakdown within PS II. Chemical uncoupling of the higher plant preparations rendered them as, if not more, sensitive to inhibition by anaerobiosis as the algal particles.

Where hydrogenase is fully active, the loss of activity in the reported reconstituted systems for H_2 photoproduction may be related to the inactivation of PS II due to the loss of the photophosphorylation coupling mechanism. The Benneman et al (1) report demonstrating short term activity included addition of NH_4Cl to the system. Where the activity reported was eventually lost due to the apparent ageing of particles (2,3) the chloroplast particles were initially prepared by a procedure that is known to promote stability and retain the coupling of photophosphorylation. Anaerobiosis is required for the protection of hydrogenase from O_2 inactivation; however, extended anaerobiosis may account for the rapid loss of PS II activity in particles which become uncoupled with time. If reconstituted systems with maximum rates and stability are to be realized, our results indicate that protection against both uncoupling of photophosphorylation and O_2 inactivation of hydrogenase is required. Whether anaerobiosis inhibits photoact II in

intact algae is an important question on the source of H_2 . Clearly photoact I is not affected as demonstrated by the process of photo-reduction.

B. Oxygen inactivation and carbon monoxide inhibition of hydrogenase from Chlamydomonas.

Oxygen is a potent inhibitor of hydrogenase. In bacteria both reversible and irreversible O_2 inhibitions have been observed. O_2 inhibition of Clostridium pasteurianum hydrogenase is rapid (50% loss of activity within two to three minutes in air) and irreversible (4). For Proteus vulgaris hydrogenase the inhibition is reversed by physical removal of O_2 (5). The mechanism proposed was that the inhibition was a simple oxygenation of the enzyme. Like C. pasteurianum, hydrogenase from Chlamydomonas rugamentosa is irreversibly inhibited (i.e., inactivated) by O_2 (6).

Carbon monoxide is another potent inhibitor of hydrogenase. For the bacterial hydrogenases from P. vulgaris, Desulfovibrio desulfuricans, and C. pasteurianum the inhibition constant is less than 30 μM (4,7). This inhibition is competitive versus H_2 and is reversible. In the enzyme from C. pasteurianum the CO molecule binds directly to an iron-sulfur cluster (8). Aside from the fact that hydrogenase from Chlamydomonas sp. is reversibly inhibited by CO little information is available on CO inhibition of algal hydrogenases (9).

In vivo and in vitro systems produce O_2 as a byproduct. Considering the O_2 lability of hydrogenase this poses some problems in the attempt to prepare long-lived H_2 producing systems. In reconstituting photolytic systems from isolated hydrogenase and photosystem particles,

the preparation of the enzyme requires a means of protection from O_2 ; otherwise, considerable loss of hydrogenase can be expected. Agents such as dithionite and glucose+ glucose oxidase have been used to scavenge O_2 from solution(1). As yet no one understands the nature of O_2 inactivation of hydrogenase. Consequently no one has been able to approach this problem from a molecular view of the hydrogenase and O_2 interaction.

We have investigated mechanisms based on reaction kinetics for the inhibition of hydrogenase by O_2 and CO. We have used CO as a molecular probe in investigating the effect of O_2 on hydrogenase. Such information will give a greater understanding of the biological catalyst responsible for H_2 production. With this knowledge new approaches to the problem of O_2 inactivation may be formulated.

Methods

C. reinhardi was grown on Tris-acetate-phosphate medium under fluorescent light and harvested at late log phase. The cell paste (200 gm) was resuspended in 800 ml of 50 mM Tris-Cl, pH 8.0, with 10 μ M DCMU and adapted under H_2 for 3 hours. Continuing under anaerobic conditions the cells were broken by freezing in liquid nitrogen and thawing at 40-50 C. $(NH_4)_2SO_4$ was dissolved in the suspension at the rate of 20 gm per liter. After centrifugation at 15,000 x g for 1 hour the supernatant was decanted, and saturated with $(NH_4)_2SO_4$ with an additional 50-70 gm per liter. The precipitated hydrogenase was recovered after centrifugation. Concentrated solutions of enzyme were prepared by dissolving the precipitate in the Tris buffer.

The enzyme was assayed by methyl viologen reduction followed on a Gilford recording spectrophotometer at 605 nm. Enzyme was injected into a cuvette with 2.0 ml of 10mM methyl viologen in 50mM Tris-Cl, pH 8.0, under 760 torr H₂ at 25 C. One unit of hydrogenase is defined as reduction of 1.0 μ mol of methyl viologen per minute.

In the O₂ studies, 1.5 ml of enzyme solution was rapidly equilibrated with O₂ in a bottle by vigorous shaking. The enzyme concentration was measured by methyl viologen reduction. Oxygen in the sample was removed by injection into assay cultures having a small quantity of reduced methyl viologen. Once the O₂ was removed no further inactivation of the hydrogenase was observed.

The inactivation of Chlamydomonas hydrogenase by O₂ was found to be irreversible. Neither removal of O₂ by repeated evacuation and H₂ or N₂ flushing nor addition of hydrogenase and H₂ or dithionite would restore activity to O₂ inactivated hydrogenase.

The reaction may be written as:

The rate of reaction (inderivative form) may be written as:

$$d(\text{H}_2\text{ase})/dt = k (\text{H}_2\text{ase})^n (\text{O}_2)^m \quad (\text{eqn 1})$$

(t, time; k, reaction rate constant; (H₂ase), hydrogenase concentration; (O₂), the concentration of O₂ in the gas phase above and in equilibrium with the hydrogenase solution; n and m, the number of reaction molecules of H₂ase and O₂, respectively).

If (O_2) in solution is much greater than (H_2ase) than eqn 1 is reduced to:

$$d(H_2ase)/dt = k_{obs} (H_2ase)^n \quad (eqn 2)$$

(k_{obs} , observed rate constant at a specific (O_2)). in which:

$$k_{obs} = k(O_2)^m \quad (eqn 3)$$

Eqn 2 can be rearranged and integrated:

$$d(H_2ase)/(H_2ase)^n = k_{obs} dt \quad (eqn 4)$$

If only one molecule of hydrogenase reacts in the rate-limiting transition complex, i.e., $n = 1$:

$$\ln (H_2ase) = k_{obs} t \quad (eqn 5)$$

The plots of $\ln (H_2ase)$ versus t are linear for (O_2) less than 0.1 atm (fig. 1 and 2). For n greater than 1 the plots are not linear. Such kinetics indicate that only one molecule of hydrogenase is involved in the reaction and not an aggregation of two or more.

Eqn can be transformed into:

$$\log k_{obs} = n \log (O_2) + \log K \quad (eqn 6)$$

The slope of the plot $\log k_{obs}$ versus $\log (O_2)$, which is approximately 1 (i.e., 0.94), gives the number of O_2 molecules participating in the reaction (Fig. 3).

CO Inhibition. CO reversibly inhibits hydrogenase. The inhibition can be removed by repeated evacuation and flushing with H_2 .

Table 3 shows a time course for removal of CO inhibition.

Influence of CO and O₂ inactivation. CO does protect the hydrogenase from O₂ inactivation as shown in Table 4.

The rate limiting step in the inactivation of hydrogenase by O₂ involves the equivalent of one hydrogenase molecule and one O₂ molecule. The kinetics do not distinguish between one O₂ molecule and two oxygen atoms reacting with the hydrogenase in the rate limiting transition complex. Since the reaction is not reversible by physical removal of the O₂ the fate of the O₂ may possibly be reduction to water or peroxide or combination with some component of the hydrogenase itself. If the Chlamydomonas hydrogenase is an iron-sulfur enzyme (as hydrogenase of other species are) then the sulfur might be removed as one of the sulfur oxides. The inability of such a strong reducing agent as dithionite to restore activity would imply that more than a simple reversible oxidation-reduction is involved.

Above 0.01 atm O₂ a second reaction interferes with O₂ inactivation (Fig 2). At 0.1 atm and 1.0 atm O₂ the rates of inactivation change with time and become slower. A possible explanation is that a number of O₂ molecules bind adjacent to the site of inactivation on the enzyme and prevent the orientation of any single O₂ molecule which is necessary to elicit inactivation of the hydrogenase. When the hydrogenase is combined with O₂ the initiation of the inactivation reaction is faster than this second reaction.

Like the bacterial hydrogenases (4,7) the C. reinhardi enzyme is reversibly inhibited by CO. Interestingly, O₂ inactivation is retarded

by the presence of a high concentration of CO. Since only one O_2 is involved in the rate-limiting step for inactivation it may be that both CO and O_2 bind at the same site on the enzyme. In C. pasteurianum hydrogenase CO binds to the iron-sulfur cluster(8). One can speculate that the site of O_2 action is also at such an iron-sulfur cluster.

This protection by CO and O_2 may be of considerable importance in preparing and storing quantities of hydrogenase. Unfortunately the CO (and its protecting quality) must be removed to provide active enzyme for biophotolytic reactors or from other studies.

C. H₂-photoevolution in intact cells

In the progress report submitted last year, we reported on the stimulatory effect of 3mM acetate on H₂-photoevolution and the concomitant drop in CO₂ formation (Fig. 4). This finding is amplified in Table 5 and indicates that glucose does not mimic acetate. Further acetate-¹⁴C did not give rise to ¹⁴CO₂ under these conditions. Label accumulated within the cells at a rate of less than 0.1 μmol per mg Chl per hr while H₂ was evolved at 25 μmol per mg Chl per hr. We propose in our progress report submitted last year that the citric acid cycle was not a source of H₂ and the reviewer of the proposal objected to our working scheme (Fig. 5). We quote from his review "in short, the results obtained thus far have not altered the picture of photoproduction proposed somewhat earlier (Plant Physiol. 45, 153 (1970)) by Healey. In this context, we note that the proposal scheme fails to account for the inhibitory effect of Krebs cycle inhibitors (monofluoroacetate)." We could find inhibition by this acid but since the ionized acid does not penetrate the cell, medium pH must be lowered to 3.5. With our strain of Chlamydomonas, pH 3.5 also inhibits respiration. Healey did not report such a control. On the other hand, Kumazawa et al(10) observed no inhibition of H₂-photoevolution in a marine blue-green alga by monofluoroacetate. The acetate explanation may well be that proposed by Heath and Leech(11) to explain the inhibitory effect of acetate and methylamine on CO₂ fixation by isolated spinach chloroplasts. These compounds affect the pH and, in turn, may act as uncouplers according to Mithcell concept. A similar suggestion was proposed by Senger and Bishop at the Madison

meeting of the American Society of Plant Physiologists. This clever idea would explain our findings in figure 4. On the other hand, we found no effect of sulfate, sulfite, nitrate and nitrite on endogenous or acetate-stimulated H_2 -photoproduction. An uncoupling action by acetate but not by glucose would speed up H_2 -photoevolution by inhibiting CO_2 formation by denying ATP for the fermentative process but releasing the rate limiting step if H_2 were derived from H_2O or a carbon-containing source not requiring ATP or releasing CO_2 such as a partially degraded fatty acid-acyl CoA derivative or the dehydrogenation of succinic acid to fumaric acid. Since the ratio of CO_2 and H_2 was 1:2 (Fig. 4), we proposed a reduced carbon compound as the source of H_2 . If the acetate explanation is correct, clearly CO_2 and H_2 are derived from different sources as suggest by Bishop and his colleagues.

Another indication that H_2 derived from endogenous and that from acetate-stimulated sources differ is provided in figure 6. The presence of 10uM DCMU inhibited H_2 -photoevolution in acetate-stimulated Chlamydomonas F-60 (a mutant lacking phosphoribulokinase) by 90% (Fig.6). In contrast, H_2 -photoevolution from endogenous sources was effected only 30 to 40% 10uM DCMU. Invoking classical dogma, (see Fig. 5) H_2O is the H_2 -donor in the acetate-stimulated cells and both photosystems are required. In sharp contrast, the bulk of the H_2 is evolved not from water but form a reduced carbon compound in the absence of acetate catalyzed only by photosystem I. It would appear that the endogenous substances inhibit the H_2O -splitting act under these anaerobic conditions.

Therefore, cells of *Scenedesmus* were starved aerobically for 60 hours to remove the bulk of the endogenous carbon material. The cells were adapted and H₂-photoevolution was nil. Once again, acetate stimulated H₂-photoevolution. Glucose, on the other hand, caused a smaller evolution of H₂ but in time approached the low endogenous level.

2. Photorespiration

A. Glycolate biosynthesis

Evidence is overwhelming that the two carbon α -hydroxyacid, glycolate, signals the entry of photosynthetically reduced carbon into what has been called the photorespiratory pathway (12). The mechanism whereby glycolate is formed from intermediate compounds of the Calvin cycle remains in dispute. Wilson and Calvin (13) proposed and Shain and Gibbs (14) described a reconstituted preparation containing fragments of spinach chloroplasts, transketolase and co-factors which was capable of rapid conversion of fructose-6-P (or any α -keto donor of dihydroxyethylthiamine pyrophosphate) to glycolate in the light.

Since glycolate-P is also seen upon the increase of concentration of O_2 , Bassham and Kirk (.15) suggested that glycolate-P could be formed by the oxidation of ribulose-1,5-diP to glycolate-P. This would be followed by the conversion of glycolate-P to glycolate mediated by the active glycolate-P phosphatase found in the chloroplast (16). However, these authors reported that the amount of glycolate-P which appeared after addition of O_2 was much less than the amount of glycolate formed in response to O_2 ; also the percentage increase with increasing O_2 was less for glycolate-P.

Recent findings that molecular oxygen and CO_2 competitively inhibit RuDP carboxylase led to the proposal that RuDP is oxidatively split on the enzyme, RuDP carboxylase by oxygen yielding glycolate-P and glycerate-3-P. The enzyme has been termed RuDP oxygenase (17) . this formulation, the process of photosynthesis and photorespiration

are linked together by the carboxylase and oxygenase of RuDP carboxylase and the relative rates of these processes can be expressed in terms of the kinetic properties of RuDP carboxylase with respect to the two mutually competitive substrates CO_2 and O_2 (17). Thus, according to these authors, the sole factors controlling the division of carbon between photosynthesis and photorespiration are the two gases.

Bassham and Kirk (18) undertook a study to assess the importance in vivo of glycolate-P as an intermediate in glycolate formation. The kinetic curves for the appearance and disappearance of RuDP, glycolate-P and glycolate, following the introduction of 100% O_2 to Chlorella previously photosynthesizing with $^{14}\text{CO}_2$ were determined. They concluded that only one-third to one-half of glycolate formation could be accounted by glycolate-P. The remaining glycolate formation was apparently the result of the oxidation of sugar monophosphates.

Glycolate metabolism is related to the Warburg effect or the inhibition of photosynthesis by O_2 . It has been well established that glycolate levels are directly related to O_2 but inversely to CO_2 (12). The formation of glycolate by either the transketolase or oxygenase mechanism can be satisfied by the concentrations of the two gases. However, the oxygenase proponents claim that the sole factors governing the synthesis of glycolate and in turn the efficiency of photosynthesis are the concentration of CO_2 and O_2 (19-21).

There is little doubt that CO_2 and O_2 are critical factors. On

the other hand, we have now found that pH can regulate the Warburg effect and glycolate formation. A recent publication by Robinson, Gibbs and Cotler (22) examined the influence of pH upon the O_2 inhibition of $^{14}CO_2$ assimilation in intact spinach chloroplasts. The severity of the Warburg effect (inhibition of CO_2 fixation by O_2) and glycolate formation increased with a rise in pH. The level of CO_2 was kept constant indicating that pH was effecting glycolate independent of the concentration of CO_2 . More recently, we have determined with the intact spinach chloroplast that pH is critical in the conversion of fructose-1,6-diP, ribose-5-P or glycerate-3-P to glycolate by the intact spinach chloroplast (Fig.8). More importantly, we recorded average maximal rates of 50 μmol glycolate/mg Chl $\cdot$ hr and in one instance, 92. Zelitch (23) noted that the rate of photorespiratory CO_2 release in C-3 plants was in the order of 76. In contrast, with CO_2 as substrate, glycolate yields in chloroplasts rarely reach 20 even under optimal conditions. We also noted that high levels of fructose-1,6-diP inhibited glycolate yields. Thus, critical to high glycolate yields is a reasonable level of intermediates of the cycle. Thus photorespiration could be controlled by decreasing these concentrations either by conversion to starch or by exporting these molecules to the cytosol.

Finally, there are ample data indicating a causal relationship between glycolate synthesis from CO_2 and the Warburg effect. We have now identified pH as another factor regulating the photorespiratory pathway. Oliver and Zelitch (24) have singled out certain amino acids which have no effect on the oxygenase reaction but, nonetheless regulate glycolate formation. In addition, in our preparations under the optimal

conditions, the stoichiometric yields of glycolate from intermediates of the Calvin cycle did not exceed 0.3. Clearly, there are many factors influencing glycolate formation in the chloroplast and subsequently, the Warburg effect and photorespiration, of which the concentration of CO_2 and O_2 are but two.

B. Structure

One of the features of C-4 plants and C-4 photosynthesis is that virtually all have a characteristic leaf anatomy termed Kranz anatomy (25). It is fair to state that C-4 photosynthesis as presently conceived requires and is dependent upon this particular anatomy, i.e. a compartmentation or sharing of photosynthesis between two cell types (bundle sheath and mesophyll). The lack of photorespiration in the C-4 plant is thought to be the result of the Kranz anatomy (26). As envisioned, the concentric arrangement of chloroplast-containing cells in C-4 plants acts either to inhibit photorespiratory CO_2 loss by concentration of CO_2 in the bundle sheath cells resulting in a diminished glycolate formation or to promote refixation of evolved CO_2 as it diffuses outwardly through the mesophyll cell. On the other hand, some workers (27, 28) have reported evidence that the Kranz anatomy is not essential to the C-4 pathway of photosynthesis. We have taken advantage of the developmental structure of the maize leaf as demonstrated by Leech (29) to test this concept. In the developing maize leaf, cells are arranged in a linear array in the growing leaf with the youngest cells at the base and oldest cells at the tip. The young tissue shows only one kind of plastid and dedifferentiation into mesophyll and bundle sheath chloroplasts is not seen until section distant from the base.

We have monitored our transverse sections in 12-day old leaves by electron microscopy. The developmental pattern is quite clearly distinct. We have now carried out preliminary studies on product labeling in the sections (Figures 9,10). Having established that each section is photosynthetically competent, experiments can now be considered with respect to photosynthesis, photorespiration and glycolate (see renewal proposal).