Effect of Light on Respiration and
Development of Photosynthetic Cells

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Martin Gibbs
Brandeis University
Waltham, Mass. 02154

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Abstract

I. Hydrogen metabolism by chlorophyll-containing cells.

Adaptation and deadaptation of unicellular algal cells to a hydrogen metabolism may involve not only hydrogenase but also the site of entry of electrons from NADH. NADPH can also serve as an electron donor in a chloroplast preparation fortified with hydrogenase but the rate of photoevolution falls off rapidly perhaps due to competition with NADP for electrons. The evolution of H_2 with reduced pyridine nucleotide as electron donor is coupled to ATP formation. Evidence is presented that soluble ferredoxin may not be the immediate donor of H_2 . Hydrogenases from Chlamydomonas and Scenedesmus have been highly purified and will be used to test the stoichiometry of NADH disappearance, ATP and H_2 formation and also whether H_2 -photoevolution in contrast to dark evolution is ferredoxin dependent. Studied with intact Chlamydomonas provide evidence that a reduced carbon compound and not water is the source of H_2 . The presence of acetate eliminates the CO_2 production. The ratio of $CO_2:H_2$ is 1:2 and evolution is blocked by inhibitors of glycolysis. Acetate has a strong effect on H_2O . Cells starved over a long period still retain photoreduction but will not evolve H_2 until glucose is added.

II. Starch breakdown by isolated intact chloroplasts.

It has been demonstrated for the first time that intact but not broken spinach chloroplasts can convert starch to glycerate-3-phosphate. The data indicate that contrary to present concepts, that chloroplasts degrade starch not amylolytically but by phosphorolytic pathway. Degradation of the polysaccharide is enhanced by 5 mM inorganic phosphate, the K_m of phosphorylase. Breakdown is blocked by iodoacetamide leading to the accumulation of triose phosphate. The messenger between the chloroplast and starch breakdown, the substrate of respiration is triose phosphate

and glycerate-3-phosphate, not free glucose as conceived. The data suggest that the chloroplast must have a mechanism of generating ATP and reoxidizing reduced pyridine nucleotide in the absence of light.

I. Hydrogen Metabolism.

A. Adaptation and deadaptation

Algae must be placed under N_2 or H_2 prior to the onset of a hydrogen metabolism. This "adaptation" phenomenon has been studied in many laboratories but remains little understood. We have reported as have others earlier that adaptation time for H_2 evolution varied with the strain of the algae and ranged from minutes in Chlamydomonas to a few hours in Chondrus and one to two days in Corallina (1). Hitherto, the explanation of "adaptation" and "deadaptation" has revolved about the enzyme hydrogenase. We would now propose on the basis of new but preliminary information that other enzymes may be involved. Figure 1 represents our present concept of the coupling of electron transport to H_2 evolution via the photosystems. We presented evidence in the previous progress report and elsewhere (2) that electrons may be supplied from exogenous donors such as NADH and dithiothreitol. By making use of the inhibitor dibromothymoquinone (DBMIB), we proposed that NADH donates, in the presence of DCMU, electrons at the level of plastoquinone and in vivo, from electrons provided by carbon metabolism or water. Donation of electrons appears to involve both photosystems since DCMU inhibits in the intact cell photoproduction by levels of 10-50%. The value depends upon the organism and the growth medium. Most investigators have assumed H_2O to be the source of H_2 as the result of DCMU inhibition.

We present the following to indicate that hydrogenase per se is not necessarily the only enzyme involved in adaptation.

(1) We reported last year that H_2 -uptake and evolution are subject to different adaptation periods. Unless the algal cell contains two isozymes of hydrogenase (and this is not proven) which adapt at different rates than the acceptor and donor molecules or their input into the H_2 -systems must differ. Presently there remains no proof of more than one hydrogenase but we have a lead into this aspect and it will be considered.

(2) Heretofore, evidence that adaptation simply meant "activation of hydrogenase" was derived from lack of H_2 -metabolism on re-entry of air to the intact cell or the isolated enzyme. We report the following experiment to indicate that deadaptation involves more than hydrogenase. Chloroplast particles taken from adapted cell and fortified with purified hydrogenase photoevolved H_2 with dithiothreitol or NADH as substrate. On the other hand, if these chloroplast particles were allowed to see air prior to addition of hydrogen, then electron donation occurred only with dithiothreitol and not with NADH. Therefore, the uneluciated pathway between NADH and the electron chain is also subject to deadaptation. The dithiol enters the chain by a non-enzymic pathway not subject to the same phenomenon. This experiment also confirms that the site of entry of NADH differs from that of the dithiol.

Clearly we do not know whether hydrogenase and the entry site are sequentially activated or subject to identical conditions. This needs further study.

B. H_2 -photoproduction using the reconstituted preparation.

We have continued to refine the preparation in order to obtain higher rates and gain further insight into the mechanism.

Brief sonication (8 sec) of anaerobically adaptated C. reinhardtii

results in a cell-free preparation capable of a light-dependent production of H_2 on addition of suitable electron donors (Table I). A longer sonication period (30 sec) caused a rapid decline in gas evolution. In the past we had extreme difficulty in demonstrating H_2 -evolution with NADPH but have found that very brief sonication is essential. NADH and dithiothreitol caused a sustained rate of gas evolution of at least one hour while evolution from NADPH was linear for about 5 minutes and then fell off rapidly. Our results show that the best donors were those compounds which cannot serve both as donors and acceptors. Thus, electron mediators of photosystem I like phenazine methosulfate apparently inhibited H_2 evolution by an electron drainage to a cyclic electron flow around photosystem I. Inhibition rather than stimulation of H_2 photoevolution by NADPH after a short period of illumination may be due to NADPH acting initially as an electron donor and subsequently serving as an electron donor in its oxidized form catalyzed by contaminating ferredoxin NADP reductase. If this is so, then we have uncovered a critical control bifurcation leading either to H_2 -evolution or CO_2 fixation. It follows that if NADP is available in the cell, H_2 evolution will cease. This latter point can now be monitored with the highly purified hydrogenase (see item D). Finally, the apparent K_m values of roughly 1 mM and 1.5 mM were calculated for NADH and dithiothreitol. These high values may well be lowered by opening-up the particles with detergent.

Figure 2 illustrates the apparent uncoupling of photophosphorylation to H_2 -evolution. This has not been determined previously. Addition of the uncoupler (FCCP) not only stimulated the rate of H_2 evolution selectively with NADH in contrast to dithiothreitol but also resulted

in a linear time course. H_2 formation in the presence of the dithiol was already enhanced presumably due to the uncoupling effect of the dithiol itself (3). The 50 minute period in H_2 evolution from NADH was apparently the period required for uncoupling phosphorylation from electron flow within the algal particles. To obtain preparations responsive to uncoupler, sonication time was limited to approximately 8 to 9 seconds. In some preliminary experiments, a molar ratio of ATP to H_2 of 0.8 ± 0.15 (five determinations) was observed. However, since we used crude preparations of Chlamydomonas hydrogenase, stoichiometric measurements of light dependent H_2 evolution, ATP formation and NADH oxidation must be considered tentative. These experiments demonstrate that H_2 evolution is coupled to energy conservation.

According to present concepts derived from comparative studies with bacteria, H_2 photoevolution is ferredoxin dependent. Repeatedly we have not observed in our light-dependent systems the need of ferredoxin. On the other hand, dark- H_2 evolution with our preparations from dithionite was ferredoxin dependent (Table II). We question the role of ferredoxin and propose rather a carrier more intimately tied to the photosystem, perhaps a bound ferredoxin. See the figure 1 ($x \rightarrow H_2$). We will consider this more closely in the proposal.

C . H_2 -photoevolution studied with the intact cell.

When algal cells are grown photoheterotrophically on acetate, H_2 -photoproduction is observed whereby the cells utilize endogenous reserves and the evolution of H_2 is not stimulated by the presence of exogenous carbon sources such as acetate. However, starved cells exhibit low rates of endogenous photoevolution of H_2 and are stimulated (F-60) by exogenous acetate. Mutant strains/deficient in Calvin cycle activity also exhibit the stimulation of H_2 photoproduction by endogenous

acetate (Fig.3). More importantly, both the endogenous and acetate-stimulated H_2 production are inhibited by the presence of inhibitors of glycolysis.

During endogenous H_2 -photoevolution, the ratio of $H_2:CO_2$ evolved is 2:1 (Fig. 4). This establishes a reduced carbon compound as a source of H_2 . Although exogenous acetate stimulates the rate of H_2 evolved, the rate of CO_2 evolution falls to zero. Using labeled acetate, we could determine that acetate is not the source of H_2 since anaerobic acetate uptake during H_2 -photoevolution is over 100 fold less than the rate of H_2 formation.

The presence of acetate seems to redirect the pathways of carbon metabolism providing electrons for H_2 -evolution. For instance, there is a cessation of CO_2 evolution (Fig. 4). In addition, whereas the presence of DCMU generally reduces in these cells the rate of H_2 -photoevolution from endogenous sources by 40-50%, the presence of DCMU with acetate results in a 90-100% inhibition (Fig. 5).

The expression of an adaptable hydrogenase by these algal cultures is closely related to the physiological state of the organism. Many reports of the presence or absence of hydrogenase activity in various organisms are based on the capacity of the organism to photoevolve hydrogen--a process requiring not only an adapted enzyme but the presence of utilizable substrates. Negative data may thus reflect an absence of either. In figure 6 are seen the results of long term, dark, aerobic starvation of an algal culture. Following starvation periods, the aliquots were adapted for the activation of hydrogenase or used directly for aerobic measurements. Four parameters were followed. 1. endogenous hydrogen photoevolution--requiring both enzyme activity and utilizable substrates; 2. dark, aerobic respiration--requiring respiratory enzymes and substrates; 3. photosynthesis--requiring an active photosystem and Calvin

cycle enzymes, bicarbonate being supplied exogenously; and 4. photoreduction--requiring only an adapted hydrogenase and Calvin cycle enzymes, the hydrogen gas and carbondioxide being supplied exogenously. The first activity to decline was hydrogen photoevolution; activity had virtually disappeared after 48 hours. At this same time, photoreductive capacity is still 100%, indicating no loss in hydrogenase activity. The further depletion of substrates with time is seen in the decline of aerobic respiration to a point of cessation after about 100 hours. Longer periods of starvation resulted in the gradual loss of photosynthetic CO_2 fixation, though not of the Calvin cycle activity as even 120 hours of starvation did not significantly affect the photoreductive capacity. Only after over 200 hours of starvation was a loss of 50% of the photoreductive capacity observed. The decline of utilizable substrates for hydrogen photoevolution during starvation points out the inability of this organism to utilize water as a source of electrons for hydrogen photoproduction in vivo. During the period where hydrogen evolution from endogenous sources has disappeared, the capacity to split water for electrons during photosynthesis is unimpaired. The inability of the system to split water and thus provide electrons for hydrogen photoevolution under our conditions is a problem worthy of further study.

Finally, in figure 7 we propose a mechanism for photoevolution of H_2 in those cells where H_2 is derived from a reduced carbon source. To obtain the observed ratio of 1 CO_2 , 2 H_2 , we propose H_2 evolution at the level of triose phosphate dehydrogenase and a coupled $\text{H}_2:\text{CO}_2$ evolution catalyzed by the phosphoroclastic reaction. This scheme would also account for the data recorded with the inhibitors.

D. Enzyme purification.

Heretofore, we have employed crude preparations of algal hydrogenase.

Other workers have utilized Chromatium or Clostridium hydrogenase. The arrival of Dr. David Erbes who was trained by Dr. R.H. Burris brought about an expertise in anaerobic technology. He has purified the extremely air-labile algal enzyme from chlorophyll, ferredoxin and enzymes of the photosynthetic carbon reduction cycle. Purification was achieved by breaking cells followed by ammonium sulfate precipitation and column chromatography. All steps must be performed under strict anaerobicity. The highly purified enzyme stable in liquid nitrogen will be used for future experiments to elucidate the pathway of H₂ metabolism (see proposal).

II. Starch degradation in chloroplasts.

The current concept of starch breakdown was derived a century ago, namely, that starch is hydrolyzed to glucose which, in turn, is degraded by the glycolytic pathway. However, after looking at the distribution of isotope after ¹⁴CO₂ fixation by chloroplasts for some ten years, it became evident that glucose-¹⁴C is never formed. Therefore, I questioned whether in the leaf the classical concept derived from experiments with seeds was valid.

(4)

Recent advances/in chloroplast metabolism indicate that carbon is removed from the photosynthetic carbon reduction cycle primarily as glycolate, triose-P, glycerate-3-P and starch. The first three compounds, in contrast to the insoluble polysaccharide, move freely from the chloroplast to the cytoplasm where they undergo further metabolism. While the subsequent metabolism of these soluble compounds have been the subject of exhaustive studies, little attention has been given to this mobilization of this major nutritional polysaccharide. An analysis of the recent literature/would indicate that the glucan is broken down within the organelle to triose-P prior to export, since glucose and hexose mono- and diphosphates

are transported to a limited extent across the chloroplast membrane. Supporting evidence for a glycolytic pathway within the organelle is the finding of a fructose-6-P kinase located within the chloroplast(7). We report here the first experiments on the dark metabolism of starch- ^{14}C in a preparation of isolated chloroplasts. To perform these experiments, spinach chloroplasts with isotope predominately in starch were prepared by taking advantage of the impermeability of the polysaccharide to the chloroplast envelope, in contrast to other compounds which become labeled during a short period of photosynthesis in $^{14}\text{CO}_2$.

Table III records the retention of the radioactive products in the reaction mixtures before and after a wash in isotonic buffer. Washing removed over 90 percent of the radioactivity, resulting in starch representing approximately half of the remaining isotope. In agreement with others, the phosphorylated sugars were retained by the chloroplasts.

Our data indicate that starch is broken down in the dark by the intact chloroplast (broken chloroplasts serving as control do not degrade the polyglucan) into hexose monophosphates (glucose and fructose), fructose-1,6-diphosphate, triose phosphate (dihydroxyacetone-P and glyceraldehyde-3-P) and glyceric acid-3-phosphate. These results establish that starch is dissimilated within the intact plastid through a series of well-known phosphorylated compounds. To the contrary, there is no evidence of amylolytic cleavage to glucose.

Figures 8 and 9 show that breakdown of the polysaccharide is dependent upon inorganic phosphate. The K_m is approximately 5 mM or nearly that of phosphorylase (starch $\rightarrow$ glucose-1-P). This high concentration of inorganic phosphate is sufficient to inhibit photosynthetic CO_2 fixation by the plastid indicating a rapid change in the light-dark transient in inorganic phosphate. Note also the accumulation of hexose monophosphate

indicating that fructose-6-P kinase is apparently rate limiting. The addition of fructose-1,6-diphosphate results in a decrease in hexose monophosphate and an increase in triose phosphate (Fig. 10). Clearly the oxidation of triose phosphate is now limiting. The addition of iodoacetamide (IAA) predictably resulting in the accumulation of triose phosphate indicating the presence of glyceraldehyde-3-P dehydrogenase (Fig. 11).

These findings open up a new concept in starch metabolism in the plant cell. With this preparation, the pathway and regulation of that pathway is ripe for study.

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Fig. 1

fig. 1 (cont)

TABLE I

Stimulation of H₂ photoevolution by reduced compounds
using sonicated preparations of *Chlamydomonas reinhardtii*

A suspension of cells containing about 0.4 mg Chl/ml in 25mM Na, K phosphate, pH 7.3; 2mM KCl; and 0.5mM MgCl₂ was flushed with N₂ or H₂ and adapted for 1 hr at room temperature in the dark. The cells were sonicated for 12 sec under H₂ at 0°C. The complete reaction mixture (3ml) contained the indicated electron donor; 10 µM DCMU and 2.85 ml of the sonicated cell suspension. The side arm of the Warburg flask carried alkaline pyrogallol.

Electron Donor	H ₂ Photoevolution µmoles/mg Chl·hr
NADH, 8mM	12.0
NADPH, 8mM	11.8
Dithiothreitol, 10mM	10.6
Dithioerythritol, 8mM	7.2
Ascorbate, 10mM and DCIP, 30 µM	6.6
Cysteine, 10mM	1.6
Glutathione, 10mM	0.5
NADH, 10mM and PMS, 7 µM	1.0
Minus electron donors	1.0
Dark control	0

Figure 2

TABLE II

 K_m values of algal and bacterial ferredoxinsfor H_2 evolution and NADP photoreduction

The reaction mixture (3ml) for dark H_2 evolution contained: 25mM Na, K phosphate, pH 7.3; 2mM $MgCl_2$, 20mM dithionite; the required concentration of ferredoxin and 9 mg/ml (2.85ml) protein of crude Chlamydomonas hydrogenase or 1 mg/ml protein of Clostridium hydrogenase prepared as described under Materials and Methods. The reaction mixture (1ml) for NADP photoreduction contained: 25mM Na, K phosphate, pH 7.3; 2mM $MgCl_2$, 2mM KCl, 0.1mM NADP, ferredoxin as required and spinach chloroplasts containing 30 μg Chl.

Reaction	Hydrogenase	Ferredoxin	Specific Activity $\mu moles/mg$ protein $\cdot hr$	K_m value μM
Dark H_2 evolution with dithionite	Chlamydomonas	Chlamydomonas	0.4	20
	Chlamydomonas	Clostridium	0.2	50
	Chlamydomonas	Chlorella	0.18	62
	Clostridium	Clostridium	0.37	160
			$\mu moles/mg$ Chl $\cdot hr$	
NADP photoreduc- tion with spinach chloroplasts	-----	Chlorella	36	0.7
	-----	Clostridium	33	0.8
	-----	Chlamydomonas	40	0.8

Figure 3

Figure 4

fig 5

Figure 6

PHOTOEVOLUTION OF HYDROGEN

F_{j-1} ?

TABLE ~~III~~

Distribution of Radioactivity in Products

	Total radioactivity	Radioactivity in					
	cpm/mg Chl x 10 ⁻⁶	Glycerate-3-P	Fructose-1,6-diP Percent of total	Glucose-P	Maltose	Starch	Others
Before washing ^a	3.39	51	23	5	3	9	6
After washing ^b	0.22	9	21	7	5	52	2

a) Chloroplasts (0.20 mg Chl/ml) photosynthesized in air for 30 min in a total volume of 16ml / 0.33M sorbitol; 50mM HEPES, pH 7.8; 2mM EDTA; 1mM each of Na₄P₂O₇, MgCl₂, MnCl₂, fructose-1,6-diP, and 4.35mM NaH¹⁴CO₂, 2μC/μmole. Photosynthesis was terminated by emptying the reaction mixture into a darkened centrifuge tube. An aliquot was assayed for total radioactivity and another was subjected to paper chromatography.

b) To 7.5ml of the reaction mixture of (a) were added 16 ml of chilled 0.33M sorbitol; 50mM HEPES, pH 7.8; 2mM EDTA and 1mM each of Na₄P₂O₇, MgCl₂, and MnCl₂, followed by centrifugation for 1.5 min at 2500 g. The pellet was resuspended in 7.5 ml of the same buffer and portions were assayed for total radioactivity and for distribution of isotope. All washing operations were carried out at 0 to 5 C. The time from the end of illumination to the sampling of the resuspended pellet was 7 min.

THE EFFECT OF WASHING
WITHOUT PHOSPHATE ON
STARCH BREAK DOWN

..... Rx MEDIA MINUS PHOSPHATE
—●— Rx MEDIA PLUS PHOSPHATE

PHOSPHATE = 5 mM

FIGURE 8

FIGURE 5

FIGURE 10

FIGURE 11

Legends for Figures

1. Scheme for light induced H₂-evolution.
2. Demonstration that H₂-photoevolution is coupled to ATP formation.
3. Effect of acetate and glycolytic inhibitors on H₂-evolution in Chlamydomonas F-60. This mutant lacks ribulose-5-P kinase resulting in an inactive carbon metabolism.
4. Ratio of CO₂:H₂ is 1:2 and effect of acetate on that ratio
5. Effect of DCMU on H₂-evolution following starvation.
6. Effect of starvation on photosynthesis, photoreduction, H₂ evolution and respiration.
7. A scheme of metabolism to account for the ratio of CO₂:H₂ depicted in figure 4.

Figures 8, 9, 10, 11. Starch breakdown in the chloroplast.

8,9	Effect of Phosphate
10	Effect of Fructose-1,6-diP
11	Effect of Iodoacetamide (IAA)

Principal Investigator

Martin Gibbs

- 1947-56 Brookhaven National Laboratory, Senior biologist.
- 1956-64 Cornell University, Professor, Department of Biochemistry.
- 1965 Brandeis University, Professor and Chairman (1966-69).
- 1952 Visiting Professor, University of Pennsylvania
- 1956 Visiting Professor, Queen's University, Canada
- 1958-59 Visiting Scientist, RIAS.
- 1963 Visiting Scientist, Carnegie Institution of Washington, Stanford University
- 1962-64;
71-74 Consultant to NSF: Metabolic Biology Panel
- 1963- Editor-in-Chief, PLANT PHYSIOLOGY
- 1965-68 Consultant to NIH; Fellowship Review Board, Biochemistry and Nutrition
- 1966-71 Editorial Board, Annual Reviews of PLANT PHYSIOLOGY
- 1967- Editorial Board, Physiologie Vegetale
- 1969-70 Consultant to NATO, Fellowship Review Board
- 1969 National Lecturer, Sigma Xi.
- 1971 American Academy of Arts and Sciences
- 1974 Visiting Professor, University of Munich
- 1974 National Academy of Sciences
- 1975 Honorary Vice-President of the XII International Botanical Congress, Leningrad, USSR.
- 1976 Chairman, Gordon Research Conference, "CO₂ Fixation by Green Plants."

David L. Erbes. B.S. 1970, University of Illinois.

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Dan Oscar King, Ph.D., 1974. Indiana University.

B.A. University of South Florida

M.A. Indiana University, 1971

Ph.D. Indiana University, 1974.

Alvarez, M.A. and D. King. Peroxidase localization, activity and isozyme patterns in the developing seedlings of Vanda. Amer. J. Botany 56, 180-186. (1969).

Dan King has investigated glycolate metabolism in Chlamydomonas reinhardtii by isolating and identifying mutations for the metabolic pathway of glycolate oxidation. He is currently studying hydrogenase systems in Chlamydomonas and Scenedesmus with particular interest in the ultimate source of electrons for producing hydrogen. He plans to investigate the source pools of electron donors for hydrogen and the role of the water splitting act as a possible source of electrons.

Dwight G. Peavey, B.S. 1970, Bates College, Lewiston, Maine

M.A. Brandeis University, 1973

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Ph.D. candidate at Brandeis University

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