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The Mechanism of Hydrogen Evolution by *Chlamydomonas moewusii*[1]

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ABSTRACT

Using manometric techniques, H_2 evolution in both darkness and light has been studied in the green alga, *Chlamydomonas moewusii*.

Hydrogen evolution in the dark is accompanied by the release of only CO_2 in manometrically detectable amounts. It is depressed by dark starvation and inhibited both by monofluoroacetic acid and by uncouplers of phosphorylation. This evidence suggests that the reaction is dependent on oxidative carbon metabolism for reductant and phosphorylation for energy to raise the reductant to a redox potential capable of reducing H^+.

Photoevolution of H_2 is also accompanied by the release of only CO_2. It is depressed by dark starvation and stimulated by acetate or a period of photosynthesis. Monofluoroacetic acid causes complete inhibition, while 3-(3,4-dichlorophenyl)-1,1-dimethylurea causes no or only slight inhibition. These results indicate that oxidative carbon metabolism is the source of reductant for the reaction. Photoevolution of H_2 does not show Emerson enhancement, and it has an action spectrum peaking at a longer wave length than that of photosynthesis. These characteristics, together with the slight effect of 3-(3,4-dichlorophenyl)-1,1-dimethylurea on the reaction, show that only system I of photosynthetic electron transport is involved in the reaction. Photoevolution of H_2 is stimulated by uncouplers; this indicates that the role of light is not to provide energy by phosphorylation. Rather, the results support an electron flow driven directly by light through system I from reductant produced in oxidative carbon metabolism to a redox potential capable of reducing H^+.

The release of H_2 by an alga was first observed in *Scenedesmus obliquus* by Gaffron and Rubin (11). The reaction occurred following a 2- to 3-hr incubation under N_2 both in darkness and, at a higher rate, in light. While the reaction in darkness was inhibited by the uncoupler DNP[3], that in the light was stimulated. This difference led Gaffron and Rubin (11) to propose that different reactions were involved in the dark and light. They suggested that the dark reaction depended on the oxidation of organic compounds for reductant, while that in the light derived reductant from the photoxidation of water. The former suggestion has been supported by the stimulatory effects both of glucose and of periods of photosynthesis of H_2 release in the dark by *S. obliquus* (11) and the dependence of H_2 release by *Chlorella pyrenoidosa* on an exogenous substrate (8). The dependence of photoevolution of H_2 on photooxidation of water has been supported by the observation of the simultaneous release of H_2 and O_2 in the light by anaerobic *Chlorella* sp. (25, 26). Further support has come from the observation by Bishop and Gaffron (4) of several correlations between the release of H_2 and O_2 by *S. obliquus*. In this alga, the simultaneous release of H_2 and O_2 in the approximate molar ratio of 2:1 was observed. Both the photoevolution of H_2 and that of O_2 were inhibited to the same extent by mutations, inhibitors of the Hill reaction, and manganese deficiency. Finally, both showed similar enhancements in combined red and far-red light. Neither MFA nor iodoacetic acid had any effect on the photoevolution of H_2 in this species.

The above results suggest that only electron transport from water through the two photosystems of photosynthesis to the release of H_2 is involved in the photoevolution of H_2 by algae. However, a few observations do not fit well into this model. The release of H_2 is accompanied by the release of CO_2 in *Chlamydomonas moewusii* without added substrate (9) and in *S. obliquus* in the presence of glucose (18). Several organic substrates stimulate the photoevolution of H_2 in *Ankistrodesmus braunii* (19). Finally, cell-free preparations of *Chlamydomonas eugametos* evolve H_2 from reduced pyridine nucleotides (1). These observations suggest that some algae may be able to obtain reductant for the photoevolution of H_2 from the oxidation of organic compounds as well as from the photooxidation of water. A study of H_2 evolution by *C. moewusii* was undertaken to evaluate this suggestion.

MATERIALS AND METHODS

Culturing. A culture of *Chlamydomonas moewusii* (Indiana University Collection No. 97) was obtained from Dr. R. A. Lewin. Stock cultures were maintained by weekly transfers in liquid medium on a rotary shaker. Experimental cultures, inoculated from the stock cultures, were grown in 1-liter Erlenmeyer flasks gently shaken on a wrist-action shaker and bubbled with air. Cultures were lighted from beneath by cool white fluorescent tubes and kept at 20°. The cultures were harvested in late exponential phase, when the cell density reached about 6×10^6 cells per ml. Both stock and experimental cultures were checked for bacterial contamination by inoculating 5 ml of medium containing 0.1% tryptone and 0.1% glucose (w/v) with 1 ml of culture.

The culture medium (FW-2) had the following composition: $NaNO_3$, 0.850 g; K_2HPO_4, 0.017 g; $MgSO_4 \cdot 7H_2O$, 0.123 g; KCl, 0.075 g; $CaCl_2 \cdot 2H_2O$, 0.074 g; tris, 1.0 g; trace element solution 1, 10.0 ml; made up to 1.0 liter with distilled water. The pH was

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[3] Abbreviations: CCCP: carbonyl cyanide *m*-chlorophenylhydrazone; DCMU: 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DNP: 2,4-dinitrophenol; MFA: monofluoroacetic acid.

adjusted to 6.8 with 1 N HCl before autoclaving. Trace element solution 1 contained the following: $FeCl_3 \cdot 6H_2O$, 490 mg; H_3BO_3, 286 mg; $MnCl_3 \cdot 4H_2O$, 180 mg; $NaMoO_4 \cdot 2H_2O$, 25.2 mg; $ZnCl_2$, 10.5 mg; $CoCl_2 \cdot 6H_2O$, 4.04 mg; $CuSO_4 \cdot 5H_2O$, 3.92 mg; Na_2EDTA, 2.23 g; made up to 1.0 liter with distilled water.

Experimental. Cultures were harvested by centrifugation (about 1000*g* for 5 min) and were resuspended in buffer FW-2. This buffer was identical with the growth medium FW-2 except for the replacement of NO_3^- and SO_4^{2-} by Cl^- on an equimolar basis and the replacement of tris buffer with 0.02 M KH_2PO_4-K_2HPO_4. The pH was adjusted by varying the ratio between the two phosphate salts. The cells were not concentrated for measurement of light-saturated photosynthesis but were concentrated about 10-fold for measurement of H_2 evolution, dark respiration, and net O_2 exchange about compensation. Chlorophyll was extracted with methanol and measured with a Cary model 14 spectrophotometer. Dry weight was determined by resuspending an aliquot of cells in distilled water, transferring them to a preweighed aluminum foil cup, and drying to constant weight at 80°.

Conventional manometric techniques were used throughout. All measurements were made at 20°. Manometers and flasks were prepared for measurement of H_2 evolution by flushing with N_2 at about 0.4 liter/min for 5 min. During routine measurement of H_2 evolution, alkaline pyrogallol (0.3 ml, prepared as in Reference 30) was placed in the center well to absorb O_2 and CO_2. Chromous chloride (0.3 ml, prepared as in Reference 22) was used to absorb O_2 during measurements of H_2 and CO_2 release. A mixture of 0.2 ml of saturated methylene blue (40 mg/ml) and 0.1 ml of a 10% palladium on charcoal suspension (250 mg/ml) was used to absorb H_2. A solution of $PdCl_2$ (prepared as in Reference 2) and a separate solution of 20% KOH were used to absorb CO. Measurements in the presence of combinations of $CrCl_2$, KOH, and Pd-methylene blue were used to identify the gases released by anaerobic cells and to determine their ratios. Mixtures of either H_2 or CO_2 in N_2 were prepared in anaerobic syringes. These were injected through side arms closed with serum stoppers into flasks which had been evacuated by the method described by Umbreit *et al.* (30).

Photosynthesis was measured under air containing about 1% CO_2 buffered with 4 M diethanolamine. The diethanolamine was prepared according to the directions of Umbreit *et al.* (30). Respiration was measured under air in the presence of 20% KOH.

Except for measurements made with monochromatic light, a set of General Electric 150-watt reflector flood bulbs was used in the manometric measurements. A filter passing only light of wave lengths longer than 600 nm was used in experiments involving CCCP to protect the chemical from possible photodestruction. Light intensity in the water bath was routinely measured with a submersible photocell calibrated against a Weston illumination meter (model 756). The energy fluxes between 400 and 720 nm of the light fields used were measured with a spectroradiometer of the type described by Bulpitt *et al.* (6). Monochromatic light was obtained by passing the light beams from one or two Kodak projectors through Balzer interference filters. The filters had maximum transmissions of 30 to 45% and half band widths of 8 to 12 nm. Monochromatic light fluxes were measured with an Eppley thermopile (linear surface with 12 bismuth silver junctions) connected to a Kiethley millimicrovoltmeter (model 149). Both the spectroradiometer and the thermopile were calibrated against a standard lamp.

Ethanol solutions of DCMU, CCCP, and DNP were diluted 100-fold upon addition. Control flasks received a comparable concentration of ethanol (1 or 2%, v/v). At no time did this affect the rates of the reactions being studied. All other additions were made as solutions in FW-2 buffer.

The accumulation of citric acid following the addition of MFA was determined by the method of Taylor (29).

The above procedures have been described in greater detail (13).

RESULTS

Experimental Conditions. The only gas released by anaerobic *C. moewusii* which accumulated in the presence of alkaline pyrogallol was H_2. This was shown by the absorption of all the gas which accumulated under these conditions by a mixture of palladium and methylene blue. No CO was detected with the use of $PdCl_2$.

Preliminary to a study of the mechanism of H_2 evolution by *C. moewusii*, a number of experiments were conducted to establish conditions which would result in high and persistent rates of the reaction in both darkness and light. In the present experiments, short adaptation periods were sufficient for the appearance of hydrogenase activity. The maximum rate of H_2 evolution in the dark at pH 5 was achieved within 10 min of

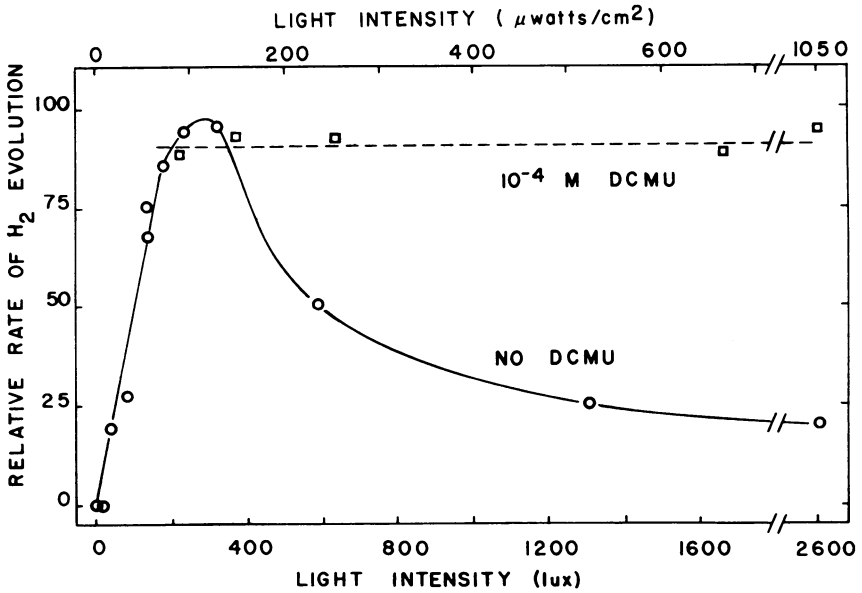

FIG. 1. The effect of light intensity on H_2 evolution by *C. moewusii* in the presence of 0.1 mM DNP at pH 6.0.

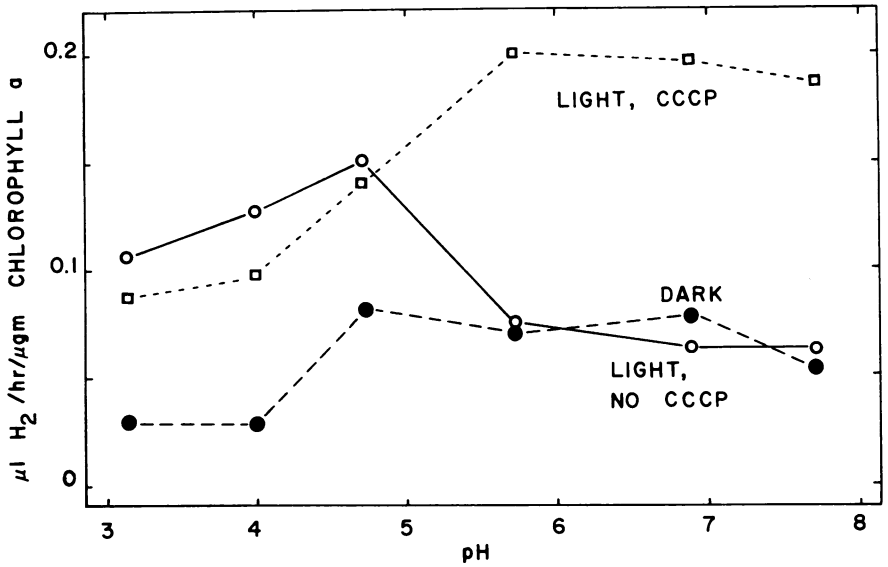

FIG. 2. The effect of pH on H_2 evolution by *C. moewusii*. White light of 9.7×10^{-5} watt/cm² (400–720 nm) was used in the absence of CCCP and red light of 1.7×10^{-4} watt/cm² (600–720 nm) was used in the presence of 30 μM CCCP.

making the cells anaerobic. This rate gradually declined with time to a small fraction of the initial rate after 4 to 5 hr. Photoevolution of H_2 at the same pH occurred at one-half or more its maximum rate within 10 min of the beginning of anaerobiosis. The maximum rate was achieved within 1.5 hr of anaerobiosis in either darkness or low intensity light (215 lux). Photoevolution of H_2 continued at its maximum rate for several hours before a slow decline began.

During the present study little effect of age of culture on rates of H_2 evolution in either darkness or light was found. Cultures from early exponential phase showed rates only slightly less than those from late exponential or early stationary phases. Light intensity during growth also had little effect on the rate of H_2 evolution in darkness. However, rates of both dark respiration and H_2 evolution in the light were up to 1.5-fold greater in cells grown at 15 klux than in those grown at 5 klux.

The effect of light intensity on the photoevolution of H_2 was measured using cells in which the dark reaction had been inhibited with DNP. Evolution of H_2 increased with intensity to saturation at about 300 lux (approximately compensation of photosynthesis). In the absence of DCMU the reaction was severely inhibited by intensities in excess of 400 lux, while in its presence, this did not occur (Fig. 1).

Cells suspended in FW-2 buffer evolved H_2 at up to twice the rate of cells in 20 mM phosphate buffer. The pH of the buffer affected H_2 evolution in the light and in the dark differently. While the reaction in the light was maximum about pH 5, that in the dark showed a broad maximum at higher pH (Fig. 2).

Inclusion of either H_2 or CO_2 in the N_2 gas phase depressed the photoevolution of H_2 by *C. moewusii*. Ten per cent of either gas in N_2 caused 30 to 40% inhibition, while 50% of either resulted in net uptake. The inhibitory effect of H_2 was largely relieved by CCCP; in the presence of 30 μM CCCP, 50% H_2 in N_2 caused only 40% inhibition of H_2 release. This suggests that the inhibitory effect of high concentrations of H_2 on net release of H_2 was largely due to the simultaneous uptake of H_2 in photoreduction.

As a result of the above findings, the following procedures were used in the experiments described below. Cultures were grown at 8 klux and harvested in late exponential phase. Buffer FW-2 was used; the pH was usually 5 in the absence of CCCP and higher in its presence. Dark anaerobic periods of 20 min and 2 hr preceeded measurement of H_2 release in the dark and in the light, respectively. Photoevolution was measured at 215 lux. When the accumulation of both H_2 and CO_2 in the light was measured, as in the determination of stoichiometry, 30 μM CCCP was added to prevent photoreduction.

Table I. *Effect of Dark Starvation Followed by Addition of Acetate or a Period of Photosynthesis at 8 klux on Reactions in C. moewusii*

Measurements were made at pH 6.0.

Treatment	H_2 Evolution		O_2 Exchange	
	Dark	215 lux	Dark	10.7 klux
	μl/mg dry wt·hr			
Not starved	2.7	4.1	11.9	115
Not starved + 1 mM acetate	2.0	4.1	13.8	115
Starved 24 hr	1.9	2.0	9.1	124
Starved 24 hr + 1 mM acetate	...	3.5	9.8	115
Starved 24 hr + 10 hr photosynthesis	2.8	4.0	12.5	120

H_2 Evolution in the Dark. Anaerobic *C. moewusii* in the dark released only CO_2 and H_2 in manometrically detectable amounts. The gases were released in the ratio of 2.2 moles of CO_2 per mole of H_2 (average of seven determinations with a standard deviation of 0.46). Dark starvation for 1 day depressed subsequent H_2 evolution in the dark; this was restored by short periods of photosynthesis. Addition of 1 mM acetate (pH 6) resulted in only inhibition of H_2 release in the dark by unstarved cells; the effect of acetate on this reaction in starved cells was not measured (Table I). Hydrogen evolution in the dark was completely inhibited both by MFA (Table II) and by the uncouplers CCCP and DNP (Table III).

H_2 Evolution in the Light. Like the dark reaction, photoevolution of H_2 was accompanied by the release of only CO_2. In the presence of 30 μM CCCP, a molar ratio of CO_2 to H_2 of 0.74 (10 determinations with a standard deviation of 0.15) was found. The release of only CO_2 with H_2 suggested that oxidative carbon metabolism was the source of reductant for the reaction. This was further tested by determining the effect of dark starvation and addition of substrate on the rate of the reaction. Dark starvation for 1 day reduced the subsequent rates of both photoevolution of H_2 and dark respiration (Table I). Addition of acetate to dark-

Table II. *Effect of Respiratory Inhibitors on Reactions in C. moewusii at pH 3.5*

Reaction	Rate after Addition (% of Control)			
	MFA, 10 mM		Iodoacetic acid, 0.1 mM	
	1st hr	3rd hr	1st hr	3rd hr
H_2 evolution (dark)	0	0	...	...
H_2 evolution (light)	0	0	72	0
Respiration (dark)	52	23	74	22
Photosynthesis	94	77	...	...

Table III. *Effect of Uncouplers and DCMU on H_2 Evolution by C. moewusii*

Conditions	Rate after Addition (% of Control)				
	CCCP (30 μM)	DNP (0.1 mM)	DCMU (0.1 mM)	0.1 mM DCMU +	
				CCCP (30 μM)	DNP (0.1 mM)
Dark, pH 7	10	0	...	...	...
Light, pH 6	...	150	110	...	340
Light, pH 7	470	...	260	510	...

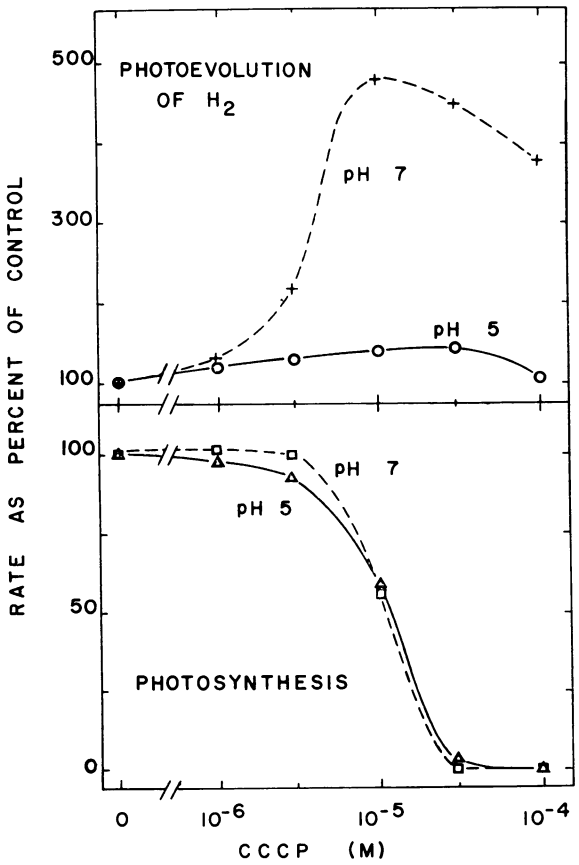

FIG. 3. The effect of CCCP on photoevolution of H_2 and photosynthesis by *C. moewusii*. Red light with intensities between 600 and 720 nm of 1.7×10^{-4} watt/cm² (photoevolution of H_2) and 5.1×10^{-4} watt/cm² (photosynthesis) was used.

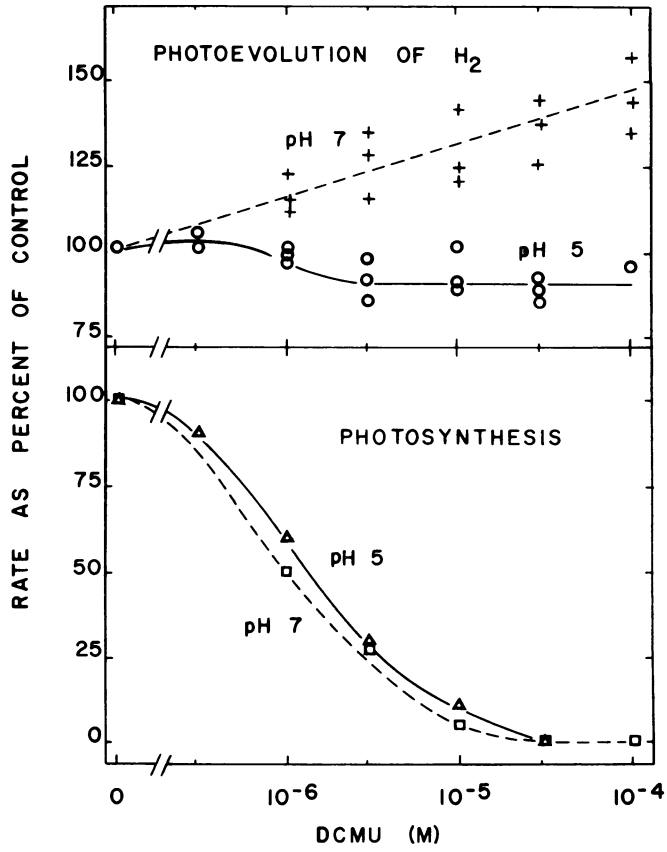

FIG. 4. The effect of DCMU on photoevolution of H_2 and photosynthesis by *C. moewusii*. White light with intensities between 400 and 720 nm of 9.7×10^{-5} watt/cm² (photoevolution of H_2) and 7.5×10^{-4} watt/cm² (photosynthesis) was used.

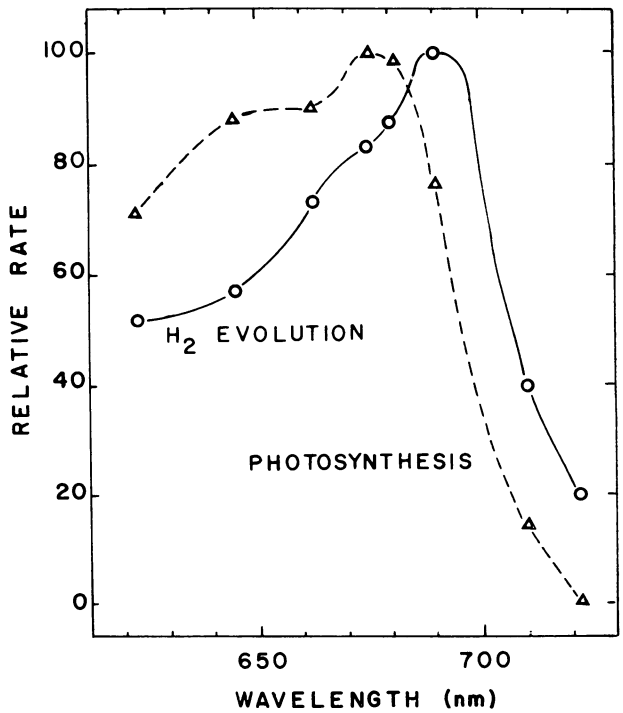

FIG. 5. Action spectra of photosynthesis and H_2 evolution by *C. moewusii*. pH 7.0. Photosynthesis was measured at 4.0×10^{14} quanta/cm²·sec. H_2 evolution was measured at 0.90×10^{14} quanta/cm²·sec in the presence of 10 μM DCMU and μM 30 CCCP.

starved cells at concentrations between 10 and 0.1 mM at pH 6 gave maximum stimulation of H_2 evolution at 1 mM; addition of the above concentrations at pH 5 resulted in inhibition increasing with concentration. While 1 mM acetate gave partial restoration of both H_2 evolution and respiration, 10 hr of photosynthesis completely restored the unstarved rates of both reactions. Light-saturated photosynthesis was unaffected by either starvation or 1 mM acetate.

To clarify further the role of oxidative carbon metabolism in the photoevolution of H_2 by *C. moewusii*, the effect of respiratory inhibitors on the reaction was measured. Monofluoroacetic acid (10 mM) completely inhibited H_2 evolution within 15 min of addition (Table II). The same concentration immediately inhibited respiration 50%, while causing only slight inhibition of photosynthesis. In each of three experiments involving inhibition of the photoevolution of H_2 with 10 mM MFA, accumulation of citric acid was detectable; an accumulation of about 0.1 μg of citric acid per mg, dry weight, was estimated. Iodoacetic acid (0.1 mM) caused 25 to 30% inhibition of both respiration and photoevolution of H_2 during the 1st hr following addition (Table II). This increased to complete inhibition of H_2 evolution in the 3rd hr and of respiration in the 4th. Malonic acid at concentrations up to 0.1 M caused only slight inhibition of both photoevolution of H_2 and respiration at pH 3.5; this inhibitor may not have entered the cells.

To determine whether light was acting directly on electron transport as in *S. obliquus* (4) or indirectly through phosphorylation as in *Rhodospirillum rubrum* (5), the effect of uncouplers on photoevolution of H_2 by *C. moewusii* was measured. At a concentration completely inhibiting photosynthesis, CCCP stimulated the photoevolution of H_2 up to 1.5-fold at pH 5 and up to 5-fold at pH 7 (Fig. 3). Inhibition of photosynthesis by CCCP was the same at pH 5 and 7 (Fig. 3), showing that the pH difference in the response of H_2 evolution to the uncoupler was not a permeability effect. The greater stimulation of H_2 evolution by CCCP at the higher pH suggested that the uncoupler might overcome the inhibitory effect of neutral pH on the reaction; that this was true is shown in Figure 2. While addition of CCCP after adaptation resulted in stimulation of H_2 release in the light, addition of 30 μM CCCP before the cells were made anaerobic prevented the appearance of H_2 evolution.

The question of the role of one or both of the photosystems of photosynthetic electron transport in the photoevolution of H_2 by *C. moewusii* was approached first with the use of DCMU. Like CCCP, the effect of DCMU on the photoevolution of H_2 was influenced by the pH of the medium. As shown in Figure 4, sufficient concentration of DCMU to completely inhibit photosynthesis inhibited the photoevolution of H_2 only 10% at pH 5 and stimulated it up to 50% at pH 7. There was no effect of pH on the inhibition of photosynthesis by DCMU. Like CCCP, DCMU caused a shift in the pH optimum of the photoevolution of H_2 to higher pH. The simultaneous addition of DCMU with either CCCP or DNP, all at concentrations giving maximum effects, resulted in greater stimulation of the photoevolution of H_2 than addition of the uncouplers alone (Table III).

The inability of DCMU to cause more than slight inhibition of the photoevolution of H_2 suggested that system II was not involved in the reaction. This was further tested by measurement of action spectra and enhancement. Action spectra and enhancement measurements of the photoevolution of H_2 were made at pH 7 in the presence of both DCMU and CCCP, conditions which yielded the highest rates. The action spectra of H_2 evolution and of photosynthesis were determined three times with similar results; the averages are compared in Figure 5. The procedure used readily detected enhancement in photosynthesis (Table IV). However, using a variety of ratios between far red and red quantum fluxes, none could be shown for H_2 evolution. Although no measurements approached light saturation, combination of wave lengths resulted in values less than 1, indicating inhibition.

Table IV. *Enhancement of Photosynthesis and Its Absence in Photoevolution of H_2 by C. moewusii*

Measurements of H_2 evolution were made in the presence of 10 μM DCMU and 30 μM CCCP.

Reaction	$Flux_{711}$	$Flux_{645}$	Ratio of Rate $_{711}/Rate_{645}$	Enhancement[1]
	quanta/cm²·sec			
H_2 evolution	81×10^{12}	86×10^{12}	0.40	0.85
	131×10^{12}	86×10^{12}	0.60	0.68
	168×10^{12}	56×10^{12}	1.48	0.86
Photosynthesis	280×10^{12}	76×10^{12}	0.45	1.9

[1] Enhancement $= \frac{\text{rate with combined wave lengths} - \text{rate at 645 nm}}{\text{rate at 711 nm}}$.

DISCUSSION

Previous studies have shown requirements for periods of anaerobiosis as short as a few minutes (28) and as long as several hours (20) for the appearance of hydrogenase activity in various algae. However, the rapid appearance of H_2 evolution in *C. moewusii* following the beginning of anaerobiosis, as well as the short-lived nature of H_2 evolution in darkness and the persistance of photoevolution of H_2 are in agreement with previous observations on this species (9, 10). On the other hand, the lack of effect of age of culture on rates of H_2 evolution found in the present study contrasts with previous observations of increasing rate of H_2 evolution with culture age in both *C. moewusii* (9) and *Rhodospirillum rubrum* (27). The present results on the effects of light intensity and gas phase on H_2 evolution are in agreement with previous observations on both *C. moewusii* (9) and *Scenedesmus obliqus* (11).

The present observations on H_2 evolution in the dark by *C. moewusii* confirm and extend previous ones on this and other algae. The release of only CO_2 and H_2, together with the depressing effect of dark starvation, suggests that oxidative carbon metabolism is the source of reductant for H_2 evolution in the dark by *C. moewusii*. This has been previously suggested for *S. obliquus* (11). The inhibition of dark evolution of H_2 by uncouplers both in *S. obliquus* (11, 4) and in *C. moewusii* indicates a dependence of the reaction on phosphorylation. Hydrogen release probably cannot be supported by phosphorylation coupled to fermentation alone because substrate level phosphorylation does not appear to be sensitive to uncouplers (24). Furthermore, the ability of MFA to inhibit completely the dark reaction indicates a dependence on the citric acid cycle. The most reasonable explanation of the above results is that phosphorylation may be coupled to electron flow from NADH produced in oxidative carbon metabolism to an unknown acceptor. This acceptor cannot be O_2, and it is probably not nitrate or sulphate because neither was added. Some high energy compound formed in phosphorylation may in turn drive electron flow from NADH to a redox level capable of reducing H^+, in a manner analogous to reversed electron flow in mitochondria (21) and chromatophores (31). The fact that H_2 evolution in the dark is usually short-lived may be due to exhaustion of the unknown electron acceptor. This mechanism is diagrammed in Figure 6.

While photooxidation of water appears to be the source of reductant for H_2 evolution in the light by *S. obliquus* (4), the insensitivity of the reaction in *C. moewusii* to DCMU shows that the latter can be supported by some other source of reductant. The release of only CO_2 with H_2 by this alga, previously reported

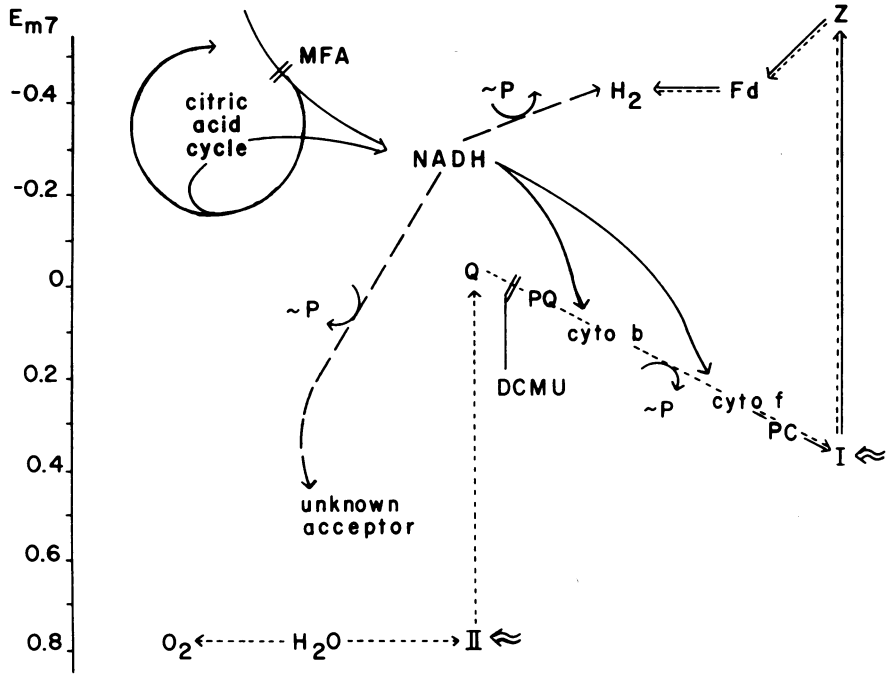

FIG. 6. Suggested mechanisms of H_2 evolution by *C. moewusii* and *S. obliquus*. ——: Pathway of reductant for the photoevolution of H_2 by *C. moewusii* in the presence of DCMU; -----: pathway of reductant for the photoevolution of H_2 by *S. obliquus* as described by Bishop and Gaffron (4); – –: electron flow during the dark release of H_2 by *C. moewusii*. Abbreviations: PQ: plastoquinone; PC: plastocyanin; Q and Z: primary acceptors of photosystems II and I, respectively; Fd: ferredoxin; ~P: high energy intermediate of phosphorylation.

by Frenkel (9) and confirmed here, suggests this source of reductant to be oxidative carbon metabolism. This conclusion is further supported by the ability of dark starvation to depress the reaction and of acetate or periods of photosynthesis to stimulate or restore it. Furthermore, the ability of MFA to inhibit completely the photoevolution of H_2 by *C. moewusii* not only confirms the involvement of the citric acid cycle in the reaction but also shows that, under the conditions used, the reaction is entirely dependent on oxidative carbon metabolism for reductant.

The stimulation, rather than inhibition, of photoevolution of H_2 by uncouplers shows that light does not act through phosphorylation to drive reversed electron flow from NADH to a potential capable of reducing H^+. Instead, the electron flow to H_2 appears to be driven directly by light. There are two possible explanations for the stimulation of photoevolution of H_2 by uncouplers. Electron transport to H_2 evolution may pass through a site of phosphorylation. In the absence of a demand for ATP, phosphorylation would impede the electron flow. By removing this impediment, uncouplers could stimulate H_2 evolution. Alternately, uptake of H_2 in photoreduction may occur simultaneously with H_2 evolution. Inhibition of the former by uncoupling would stimulate the net release of H_2. The fact that the stimulation is greater at higher pH, where photoreduction would presumably be favored by retention of CO_2 in the liquid phase, favors the latter as at least a partial explanation.

The lack of enhancement in photoevolution of H_2 by *C. moewusii* indicates that only one of the two photosystems is involved in the reaction. The lack of complete inhibition by DCMU and the action spectrum peaking at a longer wave length than that of photosynthesis show this to be system I. The inhibitory effect of combined wave lengths on the reaction remains unexplained; however, it recalls a similar effect on photoreduction in *Scenedesmus* sp. and *Ankistrodesmus* sp. (3).

While the photoevolution of H_2 by *C. moewusii* resembles that by *S. obliquus* in being stimulated by uncouplers, it differs in its insensitivity to DCMU, its sensitivity to MFA and iodoacetic acid, and its lack of enhancement (4). These differences suggest that the reaction in *C. moewusii* involves a mechanism different from that in *S. obliquus*. The mechanism proposed for the photoevolution of H_2 by *C. moewusii* is compared with that proposed by Bishop and Gaffron (4) for the reaction in *S. obliquus* in Figure 6. Although the light-dependent release of H_2 by *C. moewusii* resembles that by *R. rubrum* in being accompanied by only CO_2, stimulated by an organic substrate and inhibited by MFA, it differs sharply from the bacterial reaction in being stimulated rather than inhibited by uncouplers (12).

Although H_2 evolution both in darkness and in light appears to be dependent on oxidative carbon metabolism for reductant, the stoichiometries of gas release for the two reactions differ. This difference may be due to the different energy sources for the two reactions. As illustrated in Figure 6, the release of H_2 in darkness is dependent on NADH for both reductant and energy, while the photoevolution of H_2 is dependent on NADH for only reductant. Since CO_2 is released during the production of NADH, the CO_2 to H_2 ratio of the dark reaction would exceed that of the light reaction to the extent that NADH is used to produce energy in the dark reaction.

The slight inhibitory effect of DCMU on photoevolution of H_2 at low pH suggests a partial dependence of the reaction on system II. This would be understandable if *C. moewusii* were capable of releasing H_2 in the light from both water and oxidative carbon metabolism. However, if this were so, MFA should be able to cause only a partial inhibition of H_2 evolution, leaving intact that fraction of the reaction which could be supplied with reductant from water. The ability of MFA at low pH to inhibit completely H_2 evolution with only a slight effect on photosynthesis indicates that, under the conditions used, the cells are incapable of obtaining reductant from water. In other words, at low light intensities, anaerobiosis appears to inhibit some aspect of photosynthetic O_2 evolution in this alga. The ability of higher light intensities to reverse this inhibition is seen in the fact that at intensities above compensation the cells are apparently able to evolve sufficient O_2 to inactivate hydrogenase (Fig. 1). Evidence for an inhibition of

O_2 evolution by anaerobiosis at low intensities has been previously presented (23, 32).

In view of the uncoupling effect of high concentrations of DCMU on photophosphorylation in chloroplasts (16), the stimulation of H_2 evolution by DCMU at neutral pH could be considered analogous to that by uncouplers. However, this does not seem to be the case. Hydrogen evolution in the presence of sufficient CCCP to cause complete uncoupling of photophosphorylation (complete inhibition of photosynthesis and maximum stimulation of photoevolution of H_2) is further stimulated by DCMU. Furthermore, the stimulatory effect of DCMU occurs over the same concentration range as that causing inhibition of photosynthesis and protection of H_2 evolution at high intensity. The latter results suggest that the stimulation is due to action at the same site as that causing inhibition of O_2 evolution. The stimulatory effects both of DCMU on the photoevolution of H_2 and of 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide on the dark reduction of cytochrome *f* in *Chlamydomonas reinhardi* (14) may have a common cause. Both may be due to a possible ability of inhibitors of system II to stimulate entry of electrons into the photosynthetic electron transport system between the photosystems.

The mechanism proposed in Figure 6 suggests that *C. moewusii* should be able to evolve H_2 from reduced pyridine nucleotides in the light. Light-dependent H_2 evolution from reduced pyridine nucleotides has been observed in cell-free preparations of the closely related *Chlamydomonas eugmetos* (1). Furthermore, the mechanism suggests that reduced pyridine nucleotides should be able to feed electrons into components lying between the two photosystems. Evidence for this is seen in the ability of NADPH to reduce cytochrome b_{560} in chloroplasts (7). Further evidence for an interaction between respiratory and photosynthetic systems in *Chlamydomonas* is the observation of Hiyama *et al.* (15) that both light and O_2 cause oxidation of cytochrome b_{563} in *Chlamydomonas reinhardi*.

The mechanism proposed in Figure 6 for the photoevolution of H_2 by *C. moewusii* resembles that proposed by Jones and Myers (17) for the Kok effect in *Anacystis nidulans*. Both are based on a flow of reductant from oxidative carbon metabolism into system I of photosynthetic electron transport. All measurements of the intensity dependence of photosynthetic O_2 evolution by *C. moewusii* made during this study have shown a prominent bend about compensation. This suggests that the pathway of reductant demonstrated in anaerobic cells may be operative in aerobic cells as well.

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