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Cell-free Hydrogenase from Chlamydomonas [1, 2]

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Introduction

In 1931, Stephenson and Strickland (26) proposed that hydrogen utilization by microorganisms involved a specific enzyme. The first report of its occurrence in organisms other than the bacteria was made by Gaffron in 1939 when he discovered that certain algal strains have the ability to utlize hydrogen and CO_2 when illuminated (8). Since that time hydrogenase has been reported in Chlorophyta, Cyanophyta, Phaeophyta, Rhodophyta by Frenkel (7) and Euglenophyta by Krasna and Rittenberg (15). Recently Kanai et al. (14) have shown that the actinomycete, *Streptomyces autotrophicus,* also contains a hydrogenase. Except for the unconfirmed report by Boichenko (3) the enzyme has not been found in higher plants.

Cell-free hydrogenase has been obtained from bacteria (17). However, a comparable preparation from algae has not been reported previously. The present paper describes some characteristics of a cell-free hydrogenase obtained from Chlamydomonas.

Materials and Methods

Cultures of *Chlamydomonas eugametos* Moewus (+ strain) were grown on medium containing: 3 mmoles $NaNO_3$, 0.2 mmole $CaCl_2$, 0.6 mmole $MgSO_4$, 0.4 mmole NaCl, 0.4 mmole K_2HPO_4, 1.3 mmoles KH_2PO_4, 30.6 μmoles $ZnSO_4$, 7.31 μmoles $MnCl_2$, 6.29 μmoles $CuSO_4$, 4.96 μmoles MoO_3, 1.69 μmoles $Co(NO_3)_2$, and 67 mg ferric ammonium citrate per liter. Cultures were grown at 23±2° over a bank of 36-inch 30-w fluorescent tubes suspended 1 inch from each other and 10 cm below the bottom of the culture flasks. These flasks were shaken continuously 80 times a minute at an excursion of 5 cm and were flushed with a mixture of 5% CO_2 in air.

Cells were harvested by centrifugation and resuspended in 80 ml of disruption medium (0.125 M sucrose, 0.05 M potassium phosphate buffer pH 7.3, and 0.01 M KCl) to give an 8% by cytocrit volume suspension. This suspension was flushed with hydrogen and permitted to adapt for 1 hour at 23° before sonication with a 10 kc Raytheon magnetostrictor oscillator under argon for 3 minutes. Sonicated material was then transferred by syringe into four 13 ml Spinco centrifuge tubes fitted with gas tight aluminum caps. Unless otherwise stated, material was centrifuged for 15 minutes at 300 × *g* to remove whole cells. After centrifugation portions of the supernatant fluid were examined microscopically to insure that it contained no whole cells. Occasionally when whole cells were found in the supernatant fluid, they were removed by recentrifugation. Unless otherwise indicated, 1 ml supernatant fluid was added to each Warburg vessel.

All manipulations were carried out under conditions of strict anaerobicity since extremely small quantities of O_2 irreversibly inactivated the preparations. For example, sonication equipment, syringes, and centrifuge tubes were flushed with argon before

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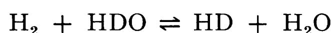
use and all transfers were made with syringes. Storage of such preparations in ice was satisfactory; only 10 to 20% of the activity was lost in 24 hours. A Gilson Medical Electronics Warburg apparatus (model RWBP3) equipped with 30-w reflector spot lamps was used for manometric measurements.

A Nier-Consolidated mass spectrometer (model 21-201) was used to monitor the gas phase of a Warburg type flask. Design and use of the equipment has been described previously (5, 13).

Results and Discussion

Exchange Reactions. It has been shown (15, 16, 24) that hydrogenase can catalyze an exchange reaction between hydrogen gas and deuterium oxide. This reaction can be represented as follows:



$$H_2 + HDO \rightleftharpoons HD + H_2O$$

The exchange reaction was measured by placing either whole cells or cell-free preparations of Chlamydomonas in 20% D_2O under H_2 and measuring the increases in mass 3 (HD) and 4 (D_2) in the gas phase with the mass spectrometer. No increase in mass 3 or 4 occurred when H_2 or HDO were omitted or when preparations were either boiled or given a prior exposure to O_2. An example of representative exchange data for a cell-free preparation of Chlamydomonas is shown in figure 1.

Hydrogenase Assays. The reduction of dyes, including methylene blue, benzylviologen, and methylviologen, have been used as assays for bacterial hydrogenase activity. In addition Tamiya (28) and Peck and Gest (22) have shown that hydrogenase preparations from bacteria can also catalyze the evolution of hydrogen from reduced methylviologen. Figure 2 presents manometric data of hydrogen uptake with methylene blue and hydrogen evolution from reduced methylviologen by aliquots of a cell-free preparation from Chlamydomonas. Both methylviologen and benzylviologen also were reduced by molecular hydrogen though the rates of gas uptake with these oxidants were one-tenth that shown for methylene blue.

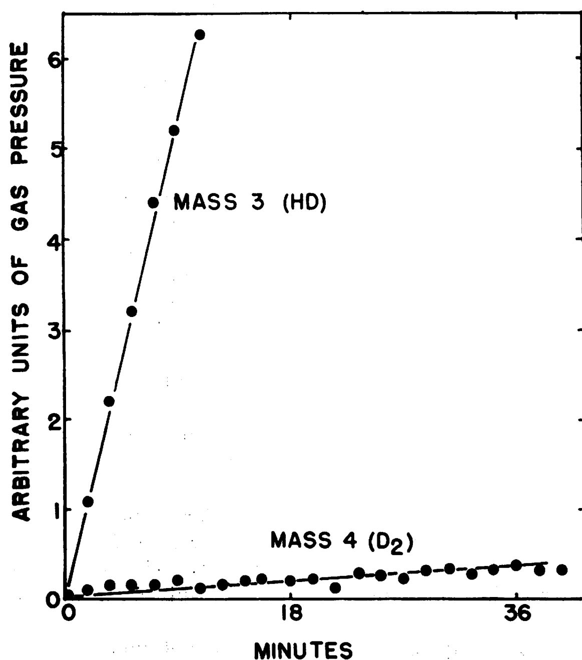


FIG. 1. Exchange reaction with hydrogenase from Chlamydomonas eugametos. 15°, 100 µmoles PO_4, pH 7.2, 250 µmoles sucrose, 20% D_2O, H_2 gas phase, 2 ml liquid volume.

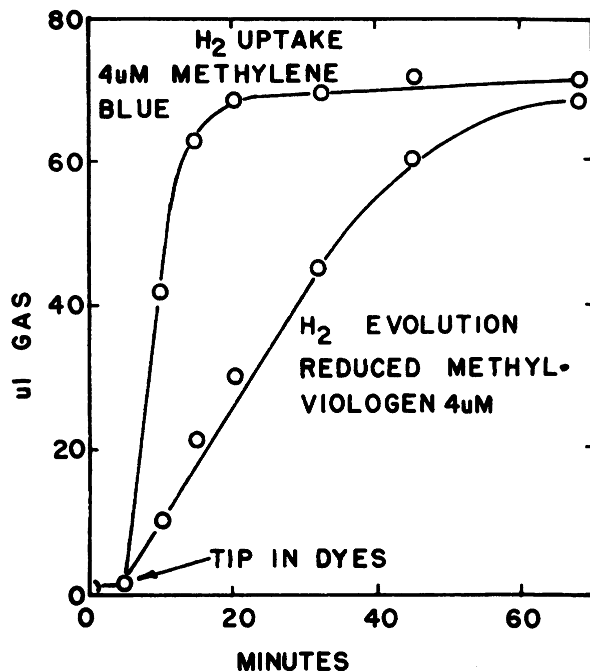


FIG. 2. Comparison of 2 hydrogenase assays. 20°, 100 µmoles PO_4, pH 7.7, 250 µmoles sucrose, H_2 gas phase for methylene blue, N_2 gas phase for reduced methylviologen, 2.2 ml liquid volume.

Attempts to use ferricyanide as an oxidant for hydrogenase assays were unsuccessful. It was found that concentrations of ferricyanide as low as 2×10^{-4} M would completely inhibit hydrogenase activity as measured by benzylviologen reduction. The reason for inhibition by ferricyanide is not known, although it is possible that it was due to oxidation by ferricyanide of the active site on the enzyme.

Oxygen Inhibition. Figure 3 shows the effect of O_2 on the rate of methylene-blue reduction. In this experiment, flasks were loaded under hydrogen with enzyme and substrate. Then, by suitable manipulation of the manometers known quantities of air were introduced to supply oxygen.

The inhibitory effect of O_2 was irreversible. Removing O_2 with a hydrogen gas flush did not restore the initial activity of the enzyme. Attempts to reactivate oxidized hydrogenase by reduced pyridine

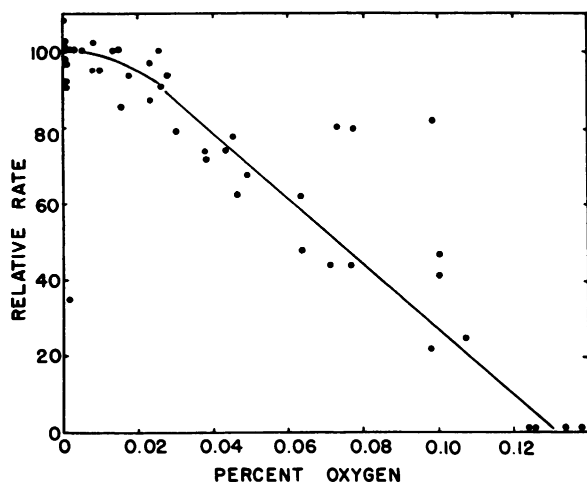


FIG. 3. Effect of O_2 on rate of hydrogen uptake during methylene blue reduction. 20°, 100 μmoles PO_4, pH 7.7, 250 μmoles sucrose, 4 μmoles methylene blue, 2 ml liquid volume.

nucleotides, ascorbic acid, reduced glutathione and cysteine, were unsuccessful.

The initial change in slope of the curve at low O_2 pressures probably is significant. The relative insensitivity of the enzyme to low O_2 concentrations may be due to the presence of a protective oxy-hydrogen reaction. However, the relatively low sensitivity of the manometric method did not permit direct determination of an oxy-hydrogen reaction since under these conditions a change of 2 to 3 mm of Brodie fluid would represent maximum gas uptake.

Because of its greater sensitivity, the mass spectrometer was used to measure the oxy-hydrogen reaction. Figure 4 gives the results of a representative experiment showing the change in mass 32 under hydrogen and helium gas phases in the presence of hydrogenase. This figure shows that there was an uptake of O_2 in the presence of hydrogen due to an oxy-hydrogen reaction. In the control experiment performed under helium the rate of O_2 uptake was significantly lower.

Temperature. Figure 5 demonstrates the effect of temperature on hydrogenase activity with methylene blue in the form of an Arrhenius plot. From these data an energy of activation of 9.8 Kcal/mole was determined. From exchange reactions, Goldsby (11) has found that the energy of activation for *Scenedesmus obliquus* hydrogenase was 7.65 Kcal. Comparable experiments with *Proteus vulgaris* hydrogenase have given values of 7.7 Kcal (12).

pH Effects. The pH optimum of methylene blue reduction by hydrogen via Chlamydomonas hydrogenase was studied using phosphate, glycylglycine, borate, and carbonate buffers. Similar results were obtained with all buffers. From pH 6 to the optimum at 9.5 the rate of reduction increased linearly. Above 9.5, however, the rate fell off sharply and at pH 10.5 there was no measurable hydrogenase activity.

Effect of Cations on Methylene Blue Reduction. Attempts to find ions which might stimulate the action of hydrogenase were unsuccessful. The following cations at a concentration of 10^{-3} M were examined for their effect on the reduction of methylene blue: Ca^{+2}, Co^{+2}, Fe^{+2}, Mg^{+4}, Mn^{+2}, Mo^{+6}, Na^{+1}, and Zn^{+2}. Of the ions tested, none stimulated hydrogenase preparations. The ions had either no effect at

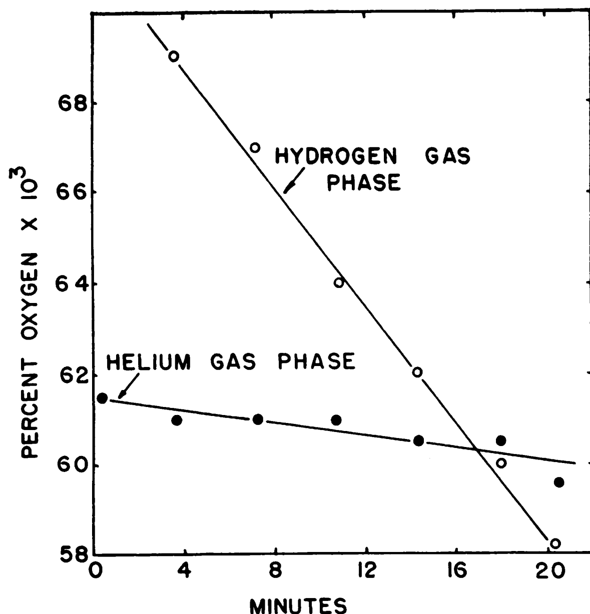


FIG. 4. Mass spectrographic determination of the oxy-hydrogen reaction with cell free preparations from *Chlamydomonas eugametos.* 15°, 100 μmoles PO_4, pH 7.7, 250 μmoles sucrose, 2 ml liquid volume.

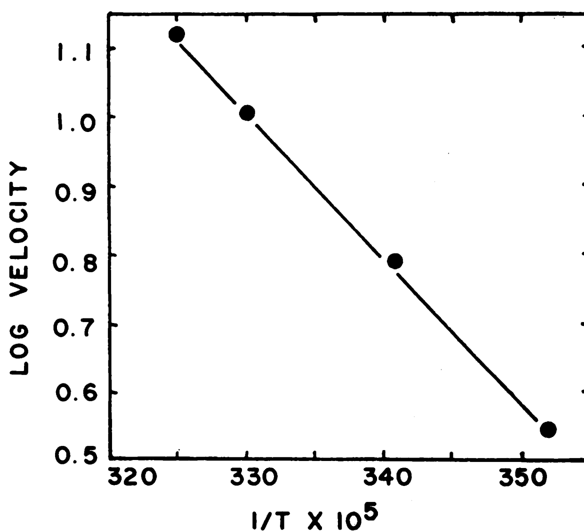


FIG. 5. Effect of temperature on rate of hydrogen uptake during methylene blue reduction. 100 μmoles PO_4, pH 7.7, 250 μmoles sucrose, 4 μmoles methylene blue, H_2 gas phase, 2 ml liquid volume.

all, or they inhibited hydrogenase action. Of the ions which inhibited, Ca and Mn had the greatest effect (about 50% inhibition).

These results indicate that none of the cations tested function as a cofactor for this enzyme. It is possible, however, that a sufficient amount of a necessary cation already is present in the crude preparations. Also, some other ion not tested may act as a cofactor. Only the effect of 10^{-3} M of a particular ion was tested. Other concentrations possibly would have given different results. This concentration was selected since equivalent amounts have been reported to give maximum stimulation with bacterial hydrogenase.

Absence of a stimulatory effect of cations on hydrogenase activity is surprising since the in vivo enzyme has been shown to be sensitive to CN and CO (9), both of which normally combine with heavy metal prosthetic groups. Hydrogenase from certain bacteria have been shown to be stimulated by molybdenum (25) and iron (21). However, Krasna et al. (17) have shown that the hydrogenase of *Desulfovibrio desulfuricans* does not have any readily dissociable cofactors, metal or otherwise.

Effect of Centrifugation on Cell-free Preparations. Cell-free preparations of Chlamydomonas were subjected to a centrifugal field of 60,000 × *g* for various periods of time to determine the relationship between hydrogenase activity and cellular fractions. Centrifugation at this force for 10 minutes almost completely removed the chloroplasts; the supernatant fluid was a clear greenish opalescent solution which showed no Hill reaction activity. The supernatant fluid contained the same amount of hydrogenase activity as the original cell free preparation. Hydrogenase activity was sedimented only after 60 minutes at 60,000 × *g*. This suggests that the enzyme is associated with particles of small size. The nature of these particles is unknown. It is possible that the method used to prepare cell-free extracts caused separation of hydrogenase containing particles from intracellular structures with which they normally are associated.

Hill Reactions. The crude cell-free preparation used in this study contained chloroplasts and chloroplast fragments which were active in catalyzing the Hill reaction. Addition of quinone, ferricyanide, or NADP to chloroplasts as oxidants resulted in Hill reaction rates comparable to those obtained with higher plants. (5–7 µl O_2/min × mg chlorophyll for NADP).

The Hill reaction of Chlamydomonas chloroplasts seemed to be similar to that observed with chloroplasts of higher plants. It was possible to observe the stimulating effect of chloride ion on the Hill reaction first noted by Warburg and Luttgens (30). The optimal rate of the Hill reaction with 0.01 M KCl was about 4 times that observed in the absence of added KCl.

The inhibiting effect of DCMU (3-(3,4-dichlorophenyl)-1,1-dimethyl urea) on O_2 evolution by the Hill reaction (2) was observed with Chlamydomonas chloroplasts. DCMU at concentrations as low as 5×10^{-5} M, completely inhibited O_2 evolution.

Hydrogenase Chloroplast Reactions. The statement that hydrogen evolution was observed when a mixture of spinach chloroplasts, bacterial hydrogenase, and ascorbate was illuminated has been made in a series of communications from Berkeley (1, 18, 20, 27). This observation of a light-dependent evolution of hydrogen is of interest because it represents a cell-free analogue of a similar reaction which occurs in adapted algae. While it may only be a physiological artifact, (the Berkeley system being composed of both bacterial and vascular plant enzymes), it does bear resemblance to the observation by Gaffron (10) that illumination of adapted *Scenedesmus obliquus* under nitrogen causes a tenfold increase in hydrogen evolution over the dark rate.

Since Chlamydomonas preparations contain both hydrogenase and chloroplasts, it was thought that this material would be well suited for duplicating the observation of the Berkeley group. An ascorbate-dependent reaction was not observed with Chlamydomonas preparations, but it is possible that requisite experimental conditions were not achieved, as the Berkeley papers furnished little specific information on materials and methods and no representative data were presented. The reaction mixture (100 µmoles PO_4, pH 7.1, 250 µmoles sucrose, 20 µmoles KCl, argon gas phase, 14°, 2.2 ml liquid volume) produced about 3 µl H_2 per 10 minutes whether ascorbate (up to 20 µmoles) was added or not.

The gas evolved by such chloroplast hydrogenase preparations was described as hydrogen by the Berkeley group although no information was given in their communications on the method of identification. To determine whether or not hydrogen was the gas evolved in my experiments, aliquots of Chlamydomonas material were studied using a mass spectrometer. Illumination of Chlamydomonas preparations caused an increase in mass 2 (H_2) and no change in masses 28 (N_2), 32 (O_2), and 44 (CO_2) over dark controls. No change occurred in any of the gas phase constituents when boiled preparations were studied. Additional confirmation was given by the observation that the gas evolved in manometric studies was not absorbed by alkaline pyrogallol.

Reduction of Pyridine Nucleotides by Hydrogen. During the course of these experiments, it was discovered that addition of NADP to Chlamydomonas preparations caused a dark uptake of hydrogen. A comparison between hydrogen uptake supported by methylene blue and by NADP is shown in table I. Use of different preparations for each experiment, explains in part, the wide variation in results. No hydrogen uptake was observed in vessels which contained no NADP or methylene blue. Not included in this table are the results of several trials in which an active hydrogenase was present (determined by methylene blue reduction), but no uptake of hydrogen was observed in the presence of NADP. Such observations demonstrate that there are factors other than hydrogenase required for electron transfer from

Table I

Relative Rates of Hydrogen Uptake With Methylene Blue and NADP

Temperature, 15° for all experiments except for last which was at 20°. 100 μmoles PO_4, pH 7.3, 250 μmoles sucrose, 20 μmoles KCl, 0.02 μmoles DCMU, H_2 gas phase, 2.2 ml liquid volume. Each experiment represents a single vessel.

Rate* meth. blue	μmoles meth. blue	Rate* NADP	μmoles NADP	Meth. blue rate / NADP rate
37	4	3	4	12
19	4	3	4	6.3
60	4	6	5.2	10
24	4	1	4.2	24
21	4	2	4	10
46	8	5	4.1	9.2
144	8	9	4	16
37	4	4	5	9
43	4	7	5	6
15	4	2	3	5
			Average	9.7

* Rate is expressed in μl gas consumed in 10 minutes.

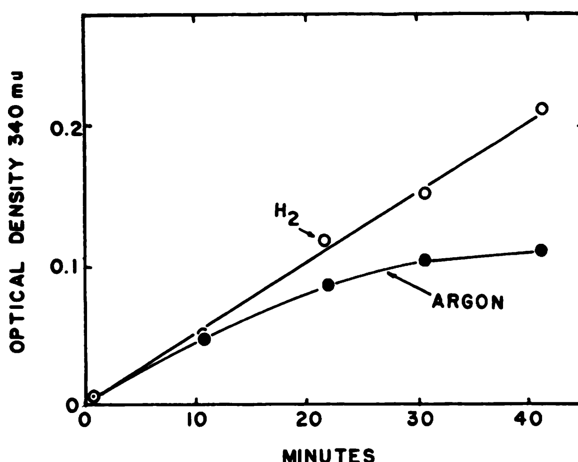


FIG. 6. Reduction of NADP under hydrogen and argon. 21°, 100 μmoles PO_4, pH 7.3, 250 μmoles sucrose, 20 μmoles KCl, 2.3 μmoles NADP, 2 ml liquid volume.

hydrogen to pyridine nucleotides. Work on ferredoxins and flavoproteins (6, 27, 29) has shown that these proteins are important in electron transport between pyridine nucleotides and hydrogen and between pyridine nucleotides and illuminated chloroplasts. It is possible that the different preparations described here varied in their content of these proteins which may have accounted for differences in electron transport efficiency.

As confirmation for the reduction of pyridine nucleotides by hydrogen, a spectrophotometric method was used to follow the reduction of NADP during hydrogen utilization. Aliquots of material from Warburg flasks were suspended in a dilute solution of NaOH (pH 10–11) and the absorbancy at 340 mμ was measured. The NADPH is relatively stable at this pH while enzymes which might decompose it are inactivated or greatly inhibited. The measurements were made as rapidly as possible as NADPH is slowly decomposed under these conditions. Control experiments with known quantities of added NADPH indicated that results obtained by this method were accurate to within 3%.

Figure 6 shows the increase in absorption at 340 mμ when NADP is added to cell-free preparations under hydrogen or argon. The results indicate that there is a hydrogen-dependent reduction of NADP as well as an endogenous reduction.

Table II presents the results of 6 experiments designed to compare rates of hydrogen uptake with NAD and NADP. According to these results NADP is reduced approximately 1.5 times as fast as NAD. Spectrophotometric experiments with NAD, with and without hydrogen, indicated that the appearance of the reduced form of NAD was strictly correlated with hydrogen uptake.

While reduction of pyridine nucleotides by molecular hydrogen has not previously been reported for algal systems such observations are not surprising in view of results published on bacterial enzymes. For example, Repaske (23), Wittenberger and Repaske (31), and Packer and Vishniac (19) have reported the reduction of pyridine nucleotides by hydrogen via bacterial hydrogenases. As yet, it has not been possible to observe a reversal of this dark reduction, i.e., net hydrogen evolution in the dark from reduced NAD or NADP.

Photoevolution of Hydrogen by Cell-free Chlamydomonas Preparations. Although dark evolution of hydrogen was not observed using Chlamydomonas preparations supplemented with reduced pyridine nucleotides, illumination of such preparations resulted in a significant evolution of gas measured either manometrically (fig 7) or by the mass spectrometer (fig 8). These preparations have the ability to produce O_2 in the light in the presence of oxidized pyridine

Table II

Comparison of NADP and NAD Reduction as Measured by Hydrogen Gas Uptake

Temperature, 20°, 100 μmoles PO_4, pH 7.3, 250 μmoles sucrose, 20 μmoles KCl, 0.02 μmole DCMU 3 mg pyridine nucleotide, all experiments except first set 0.1 ml 40% KOH in center well, 2.2 ml liquid volume. Each experimental value represents a single vessel.

Rate* NADP	Rate* NAD	Rate NADP/Rate NAD
9	9	1.0
19	16	1.2
23	13	1.8
11	17	0.7
12	16	0.8
36	13	2.8
22	11	2.0
		Average 1.5

* Rate is expressed as μl H_2 consumed per hour.

nucleotides; therefore, DCMU was added to all of the reaction mixtures to prevent inactivation of the hydrogenase by O_2. Light-dependent hydrogen evolution was obtained with both NADH and NADPH

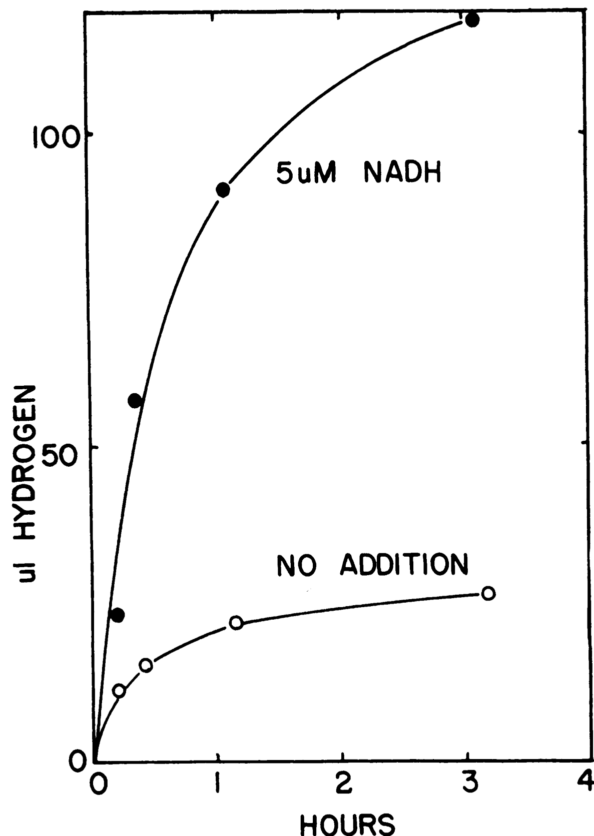


FIG. 7. Photoevolution of hydrogren from cell-free preparations of *Chlamydomonas eugametos*. 15°, 100 µmoles PO_4, pH 7.2, 250 µmoles sucrose, 20 µmoles KCl, 0.02 µmoles DCMU, He gas phase, 2.2 ml liquid volume.

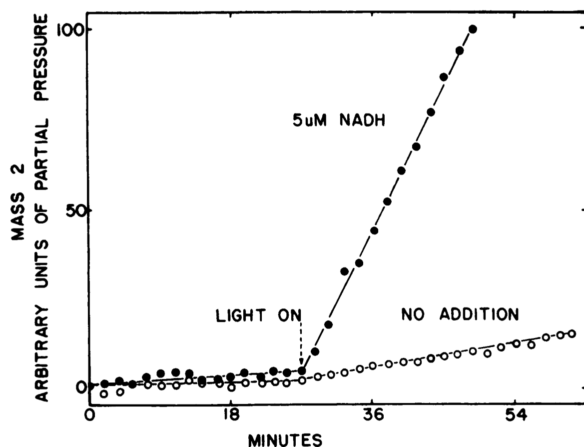


FIG. 8. Mass spectrographic measurement of hydrogen photoevolution from cell-free preparations of *Chlamydomonas eugametos*. 15°, 100 µmoles PO_4, pH 7.2, 250 µmoles sucrose, 20 µmoles KCl, 0.02 µmoles DCMU, He gas phase, 2 ml liquid volume.

Table III

Reduced Pyridine Nucleotide Stimulation of Hydrogen Photoevolution from Cell-free Preparations of Chlamydomonas eugametos

Temperature, 15°, 100 µmoles PO_4, pH 7.2, 250 µmoles sucrose, 20 µmoles KCl, 0.02 µmoles DCMU, He gas phase, 2.2 ml liquid volume.

Addition	µmoles	µl H_2 evolved/10 min
NADH	3	8
NADH	5	12
NADH	5	12
NADH	5	6
NADPH	3	7
NADPH	5	7
NADPH	5	7
NADPH	5	11
None		4
None		2

(table III). When gas evolution had essentially stopped, spectrophotometric examination of the suspension demonstrated the presence of only the oxidized form of the nucleotide.

Figure 9 presents some of the foregoing observations in the form of time course data. In the dark, both NAD and NADP caused an uptake of hydrogen during their reduction. The reason for a measurable hydrogen uptake by the control in this experiment is not known. In 3 out of 5 experiments, no hydrogen uptake was observed in the controls. After 3 hours the lights were turned on and hydrogen was evolved. Since a supply of reduced pyridine nucleotides was made available by the dark reduction, rates of hydrogen evolution in the curves marked NAD and NADP are much greater than in the control. The presence of small amounts of endogenous pyridine

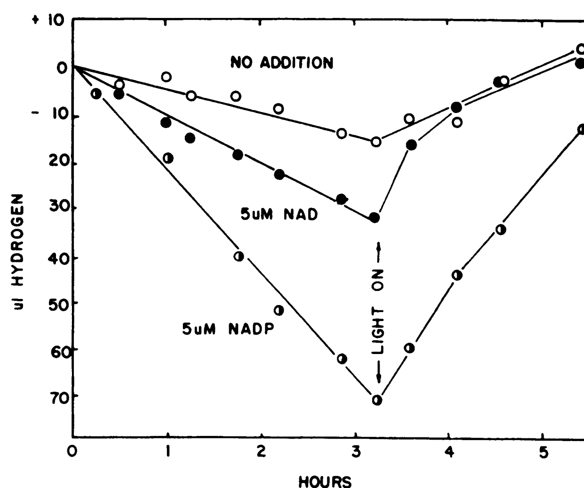


FIG. 9. Dark consumption and photoevolution of hydrogen by cell-free preparations of *Chlamydomonas eugametos*. 15°, 100 µmoles PO_4, pH 7.2, 250 µmoles sucrose, 20 µmoles KCl, 0.02 µmoles DCMU, 0.1 ml 10% KOH in center well, H_2 gas phase, 2.2 ml liquid volume.

nucleotides in crude preparations could account for the evolution of hydrogen in the control.

On the assumption that the photolysis of water into photooxidant and photoreductant is an integral part of photosynthesis, the following interpretation is consistant with the results described above. Reduced pyridine nucleotide, formed by a dark reduction, reacts with the photooxidant to form water while hydrogen is evolved from the photoreductant by means of the hydrogenase. It is likely that ferredoxin and flavoproteins act as electron carriers in these reactions, but this remains unproven.

Another interpretation of these results was given by Bose and Gest (4) who suggest that ATP, or precursors of ATP, generated by photophosphorylation could promote dark hydrogen evolution from reduced pyridine nucleotides. Agents known to inhibit photophosphorylation (antimycin *A* or 2,4-dinitrophoenol) might be used on algal chloroplast hydrogenase preparations to test this hypothesis.

Most schemes used to depict the reactions involved in photoreduction show a direct reduction of the photooxidant with hydrogen via a hydrogenase to form water. From information now available, it appears that a pyridine nucleotide could serve as an electron-transferring agent between reduced hydrogenase and the photooxidant. As my experiments have shown, pyridine nucleotides can be reduced by hydrogen gas. A photooxidation of reduced pyridine nucleotides is a likely reaction, and as mentioned earlier, has been observed with preparations from adapted Chlamydomonas by spectroscopic means. Whether such an oxidation is due to reaction between reduced pyridine nucleotides and the photooxidant or some compound oxidized by the photooxidant is not known.

Summary

It was possible to obtain active cell-free hydrogenase preparations from anaerobically adapted cells of *Chlamydomonas eugametos* (+ strain) Moewus by means of a sonic oscillation.

The hydrogenase was not associated with the chloroplast but rather with particles that were sedimented only after 1 hour centrifugation at 60,000 × *g*.

The hydrogenase was irreversibly inhibited by small amounts of oxygen or by ferricyanide. Treatment of preparations with reducing agents, or flushing with hydrogen in the case of oxygen inhibition did not restore the activity of inactivated preparations.

It was possible to reduce the following compounds by means of the hydrogenase: oxygen, methylene blue, benzylviologen, methylviologen, triphenyl tetrazolium chloride, NAD and NADP. The optimum pH was 9.5 using methylene blue as the electron acceptor.

Hydrogen evolution was observed when the hydrogenase was incubated with reduced methylviologen.

Cell-free preparations of the hydrogenase catalyzed an exchange reaction between heavy water and hydrogen.

An energy of activation of 9.8 Kcal/mole was determined for Chlamydomonas hydrogenase in vitro.

It was not possible to stimulate hydrogenase activity measured by methylene-blue reduction by addition of various cations.

An ascorbate-dependent hydrogen evolution was not observed when a mixture of chloroplasts, hydrogenase, and ascorbate were illuminated.

A reduced pyridine nucleotide dependent hydrogen evolution was observed when cell-free preparations of Chlamydomonas were illuminated. During this light dependent hydrogen evolution, the reduced pyridine nucleotides were oxidized.

Acknowledgment

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Studies of Sulfate Utilization by Algae
II. Further Identification of Reduced Compounds Formed from Sulfate by Chlorella [1, 2]

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Our previous work (3, 4) established the conditions for sulfate reduction by intact cells of *Chlorella pyrenoidosa* and resulted in the identification of adenosine-3′ phosphate-5′ phosphosulfate and *S*-adenosyl methionine as products formed from sulfate. The formation of these compounds was confirmed by Wedding and Black (6) and Cantoni (1). Iodoacetamide was employed in our earlier work to inhibit the rapid reduction process at various stages and to improve the reproducibility of the chromatographic patterns. This is based on the assumption that iodoacetamide would act both by inhibiting sulfhydryl enzymes concerned with sulfate reduction and by reacting with sulfhydryl compounds produced from sulfate preventing mixed oxidation-reduction reactions among them during isolation and separation. This paper describes the acetamide derivatives of sulfhydryl compounds which are formed from sulfate when the cells are exposed to iodoacetamide.

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Materials and Methods

The methods used for growth, incubation, and extraction of the organism are essentially the same as before (4) as are the procedures for radioactive counting, paper chromatography, and radioautog-