

FINAL REPORT  
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SURVEY OF HYDROGENASE ACTIVITY IN ALGAE

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## SUMMARY

The capacity for hydrogen gas production was examined in nearly 100 strains of Eukaryotic algae. Each strain was assessed for rate of  $H_2$  production in darkness, at compensating light intensity and at saturating light intensity. Maximum  $H_2$  yield on illumination and sensitivity to molecular oxygen were also measured. These analyses were performed in 3 and 20 hr induced cultures. Highest percentages of  $H_2$ -positive strains were found among the Chlorophycophyta. One strain produced over 100  $\mu$  moles  $H_2$  per mg chlorophyll per hr. Another strain could be induced to begin  $H_2$  production subsequent to only 4 min anaerobic incubation. A third strain maintained high rates of  $H_2$  production in the presence of 10% of atmospheric  $O_2$ . Thus, several strains showed excellent promise for future studies of algae which might be modified to sustain high steady-state rates of  $H_2$  production. Among the strains which produced  $H_2$  there was an extreme variation in the maximum rates achievable, the relative light and dark rates, and the time required for induction.

Statistical analysis of the data revealed a strong linear correlation between the initial velocity of  $H_2$  photoproduction and total amount of  $H_2$  generated in saturating light. This suggests that sustained steady-state  $H_2$  photoproduction may be impossible, but depends on a nonrenewable substrate which is depleted during photoproduction. From this and other observations it is concluded that experimental manipulation of the hydrogenase system in order to maximize  $H_2$  production cannot be efficiently attempted without a much better understanding of the biochemistry underlying the process.

## EXPERIMENTAL METHODS

All algal cultures were selected from the Culture Collection of Algae at the University of Texas at Austin. The majority of these cultures had never been grown to heavy suspension, so suitable medium had to be developed which would permit growth to approximately 10  $\mu\text{g}$  of chlorophyll per ml, using a large number of very different strains of algae. It was desirable to use the same growth medium for all strains tested since hydrogenase activity is apparently influenced greatly by culture conditions of the algae. The medium chosen was one developed in this laboratory for the hydrogenase survey. Virtually all of the results reported herein are from algae grown to a concentration of 5 to 15  $\mu\text{g}$  chlorophyll per ml in the medium listed below (Table 1). A few cultures required an organic supplement. For these the standard culture medium was modified by the addition of 10 ml of a 13.6 g/100 ml sodium acetate solution per 1,000 ml of culture medium.

Slants or dilute suspensions of cultures from the collection were inoculated axenically into 125 ml Erlenmeyer flasks containing 40 ml of culture medium. There were placed on a shaker table (1.5 cycle/sec) and allowed to grow under continuous illumination at room temperature. Light intensity (cool white fluorescent lamps) varied from 20 to 80  $\mu$  Einsteins  $\text{sec}^{-1} \text{m}^{-1}$ , depending on the shaker table used. Temperature varied from 21 to 26 degrees C.

Cultures grown to greater than 5  $\mu\text{g}/\text{ml}$  of chlorophyll were prepared for hydrogenase assays. They were first examined by light microscopy to assure the right species was present. Dr. Jeff Zeikus, who maintains the culture collection, was consulted when careful identification was required. This examination also detected microbial contamination; cultures which were clearly contaminated were rejected. Chlorophyll concentration was determined in methanol by standard methods. Suitable cultures were centrifuged at room temperature at 1,500  $\times g$  for 10 minutes to pellet cells. Fresh culture medium was added to the pellet to give a final chlorophyll concentration of 10  $\mu\text{g}$  per ml. Cultures were transferred to a 60 ml serum bottle, sealed with a rubber serum cap, then alternately evacuated and bubbled with argon for 5 cycles. Slight positive pressure was maintained in the serum bottle, which was kept at room temperature in darkness until assays were performed.

After appropriate induction times, aliquots of the cells under argon were transferred to the assay chamber. This chamber was equipped with two YSI model 5331 Clark-type electrodes. One functioned as an oxygen detector; the other was reversed in polarity and served as a hydrogen gas detector. Oxygen and hydrogen gas concentrations were both monitored continuously with calibrated strip chart recorders. The assay chamber was maintained at 25<sup>o</sup> C for all assays. A tungsten-halogen lamp equipped with an infra-red filter provided white light to the assay chamber. The compensating light intensity (see below) was 6  $\mu$  Einsteins  $\text{m}^{-1} \text{sec}^{-1}$ ; saturating light intensity was 750  $\mu$  Einsteins  $\text{m}^{-1} \text{sec}^{-1}$ .

A standard assay protocol was established whereby each strain of algae was examined by several criteria for evidence of active hydrogenase. A complete analysis for each strain was performed at 3 and at 20 hour induction. For analysis an aliquot of induced cells was transferred anaerobically by hypodermic syringe to the assay chamber. Any  $\text{H}_2$  present was recorded as concentration of  $\text{H}_2$  accumulated

TABLE I

## CULTURE MEDIUM FOR HYDROGENASE ASSAYS

To approximately 850 ml  $\delta\text{H}_2\text{O}$  add 1.95 g MES. When dissolved, add the stock solutions in the amounts indicated below. Then adjust the pH to 6.75 at room temperature using a 1 N NaOH solution. Finally bring the total volume to 1000 ml. This solution may be refrigerated for several weeks prior to use. It may be autoclaved (no more than 15 minutes at sterilization temp./pressure, to preserve the vitamins), then stored with minimum exposure to light for several weeks at room temperature.

Component	STOCK SOLUTION	
	Stock Concentration (g/100 ml)	Volume Added (ml)
$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	5	1
$\text{NH}_4\text{HCO}_3$	2	1
$\text{KH}_2\text{PO}_4$	1.5	1
Stock mix	see below	1
Thiamine	0.02	1
Biotin	$2.5 \times 10^{-5}$	1
Cobalamin	$1.5 \times 10^{-5}$	1
Spiked P IV metals	see below	6

Stock mix contains:

$\text{KNO}_3$	5 g/100 ml
$\text{MgSO}_4$	3 g/100 ml
KCl	2 g/100 ml

Spiked P IV metals is prepared as follows:

Add to 1000 ml  $\delta\text{H}_2\text{O}$  each of the following components in the order listed and amount indicated. Allow each component to dissolve before adding the next.

$\text{Na}_2\text{EDTA}$	0.75 g
$\text{FeCl}_3 \cdot \text{H}_2\text{O}$	0.097 g
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	0.041 g
$\text{ZnCl}_2$	0.005 g
$\text{CoCl}_2$	0.002 g
$\text{Na}_2\text{MoO}_4$	0.004 g
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	$1 \times 10^{-5}$ g
$\text{NH}_4\text{VO}_3$	$1 \times 10^{-5}$ g
$\text{H}_3\text{BO}_4$	$1 \times 10^{-4}$ g

in the serum bottle during induction. An examination of the  $O_2$  monitor revealed if air had leaked in during induction. The chamber was maintained in darkness until a steady-state rate of any dark production of  $H_2$  could be measured. After recording this rate, the cells were illuminated with just enough light that no  $O_2$  evolution could be detected. With our detector we could easily measure any rate of  $O_2$  production above  $5 \times 10^{-4}$   $\mu$  moles per minute in the 1.8 ml chamber. The maximum rate of  $H_2$  production with no accompanying  $O_2$  evolution (called compensation rate) occurred at almost exactly the same light intensity for the first 30 cultures tested. Thereafter, this light intensity (see above) was maintained for all compensation measurements. As soon as a steady-state rate was established (within 1-2 minutes) the illumination was increased until further increase did not improve the rate of  $H_2$  production. After determining this intensity for the first 30 cultures, a standard intensity (see above) was established which was assumed above saturation for all cultures. This illumination was maintained until  $H_2$  production ceased. The maximum rate (called saturation rate) of  $H_2$  production was recorded, as was the total amount of hydrogen produced (called total amount) during the entire illumination period. When the cells were illuminated with bright light  $O_2$  was evolved after a lag which often lasted 1-2 minutes. The maximum steady-state rate of  $O_2$  evolution was then recorded as a measure of the general health of the cells.

Cultures which had a substantial rate of  $H_2$  production were tested for sensitivity to molecular  $O_2$ . For these measurements a fresh aliquot of induced cells was injected anaerobically into the assay chamber, followed by an injection of air-saturated culture medium, in order to make the culture 2% of air saturation. Normally, a rapid  $O_2$  and  $H_2$  uptake was seen (the oxyhydrogen reaction) until all  $O_2$  was depleted. The dark rate, compensation rate, saturation rate, and total amount of  $H_2$  produced were then recorded successively just as was done for the culture without added  $O_2$ . In most cultures containing hydrogenase activity, 2%  $O_2$  substantially diminished all values obtained for  $H_2$  production. Depending on the extent of inhibition of the saturation rate of  $H_2$  production, a second injection of cells and air saturated medium was performed, giving 1%, 5% or 10% of air saturation in the cell suspension. The complete set of measurements was performed for this suspension as well. With two sets of measurements for oxygen-sensitivity, it was possible to estimate fairly accurately what concentration  $O_2$  in solution was enough to half-inhibit hydrogenase activity, as determined by saturation rates or any of the other measured rates.

In summary, for each variety of algae tested the following data were obtained:

- a. Growth medium in which cells were cultured and assayed.
- b. Chlorophyll concentration of cells when induction started.
- c. Concentration ( $\mu M$ )  $H_2$  accumulated in the serum bottle after 3 hour induction.
- d. Dark rate of  $H_2$  production ( $\mu$  moles  $H_2 \cdot mg$  chlorophyll $^{-1}$  hr $^{-1}$ ) after 3 hour induction.
- e. Compensation rate of  $H_2$  production ( $\mu$  moles  $H_2 \cdot mg$  chlorophyll $^{-1}$  hr $^{-1}$ ) after 3 hour induction.
- f. Saturation rate of  $H_2$  production ( $\mu$  moles  $H_2 \cdot mg$  chlorophyll $^{-1}$  hr $^{-1}$ ) after 3 hour induction.
- g. Total amount of  $H_2$  photoproduction ( $\mu$  moles  $H_2 \cdot mg$  chlorophyll) after 3 hour induction.

- h. Rate of O<sub>2</sub> evolution ( $\mu$  moles  $\cdot$  mg chlorophyll<sup>-1</sup> hr<sup>-1</sup>) after 3 hour induction.
- i. Repeat of parts d, e and f in the presence of 2% of air-saturating O<sub>2</sub>.
- j. Repeat of parts d, e and f in the presence of one other O<sub>2</sub> concentration besides 2% of air-saturating O<sub>2</sub>.
- k. Complete repeat of parts c-j above, except after 20 hour induction time.

For the first 40 organisms tested this complete procedure was not standardized, so some data were missing. Some of these were repeated, however, at a later date. Thus, several thousand data points were recorded in this study, with essentially complete data for 94 strains of eukaryotic algae. The volume of data obtained was so extensive that computer analysis was used to organize the information and look for interesting trends. The analysis was performed using the standard Statistical Analysis System (SAS) software developed for the IBM 370 computer, and available at the University of Texas at Austin.

## RESULTS

Complete, or nearly complete data were obtained for 94 strains of algae as applied to the experimental protocol. A second analysis was performed on about 25 of the tested strains and results varied by as much as 30% in separate experiments. A summary of the results obtained is presented in Table II. When duplicate analyses were performed the average was calculated for inclusion into Table II. From this table it is evident that the survey included mostly green algae. Initially, several divisions were examined, but most others had no demonstrable H<sub>2</sub> production. When we examined representative strains from several orders of Chlorophycophyta it became evident that active strains in some groups had much higher rates than in others. Thus, as the survey progressed, we concentrated on taxonomic groups which appeared "best" (see below for explanation of "best").

General summaries of taxonomic groups which had the largest number of active strains among those tested are included in Tables III-V. Since rates were highest in active strains from the Chlorellales and Volvocales, these were examined in most detail. Every genus within the Volvocales which was examined included strains which evolved H<sub>2</sub> subsequent to induction (Table IV). Chlamydomonas has been used in a number of previous studies of hydrogen production, and our initial sampling included several very active strains; thus, we examined many strains within this genus (Table V). Several observations are of special interest. The highest rate of H<sub>2</sub> photoproduction occurred in Chlamydomonas moewusii (2018), which evolved over 100  $\mu$  moles H<sub>2</sub> (mg chl)<sup>-1</sup> hr<sup>-1</sup> for several minutes. This represents one of the highest rates of H<sub>2</sub> photoproduction recorded in a eukaryotic alga. Altogether 5 strains of C. moewusii were tested for activity. Furthermore, two strains of C. eugametos were examined, which appeared morphologically identical to C. moewusii and may belong in the same species (R. Starr, personal communication). Rates differed by more than an order of magnitude among these strains which suggests that pronounced biochemical differences may exist among strains with respect to H<sub>2</sub> production where other differences are not apparent.

C. hydra had the lowest detectable saturation rate of H<sub>2</sub> photoproduction of any organism tested, with activity just above minimum detection level. This may

TABLE II

## SUMMARY OF ALGAL STRAINS TESTED AND ACTIVITIES MEASURES

DIVISION	ORDER	GENUS	SPECIES (UTEX NO) <sup>1</sup>	INDUCTION <sup>2</sup> TIME PRIOR TO ASSAY	DARK <sup>3</sup> RATE	COMP <sup>4</sup> RATE	SAT <sup>5</sup> RATE	% O <sub>2</sub> <sup>6</sup> FOR INHIB	O <sub>2</sub> <sup>7</sup> RATE
CHLOROPHYCOPHYTA	CHLORELLALES	ANKISTRODESMUS							
			ANGUSTUS (188)	20	0	1.8	4.8	-	-
			ARCUATUS (1379)	0					+
			BRAUNII (245)	3	0	0.3	6.5	2	+
			FALCATUS (749)	0					+
			NANOSELENE (243)	0					+
			PSEUDOBRAUNII (1380)	0					+
			SPIRALIS (107)	0					0
		BOTRYOCOCCUS							
			BRAUNII (572)	0					+
		CHLORELLA							
			PYRENOIDOSA (252)	3	0.4	4.4	31.3	1	+
			PYRENOIDOSA (1230)	0					+
		EREMOSPHAERA							
			GIGAS (122)	0					+
		GOLENKINIA							
			SP ? (931)	20	0	4.0	-	1	-
		SCENEDESMUS							
			OBLIQUUS (1450)	20	0	14.1	16.3	1	+
			OBLIQUUS (78)	20	0	0.3	9.0	1	
			OBLIQUUS (393)	20	1.4	12.1	-	2	+
			QUADRICAUDA (614)	0					+
		SELENASTRUM							
			CAPRICORNUTUM (1648)	0					+
		TETRAEDRON							
			BITRIDENS (120)	20	0.5	2.0	10.8	1	+

TABLE II, continued.

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DIVISION	ORDER	GENUS	SPECIES (UTEX NO) <sup>1</sup>	INDUCTION <sup>2</sup> TIME PRIOR TO ASSAY	DARK <sup>3</sup> RATE	COMP <sup>4</sup> RATE	SAT <sup>5</sup> RATE	% O <sub>2</sub> <sup>6</sup> FOR INHIB	O <sub>2</sub> <sup>7</sup> RATE
OEDOGONIALES									
BULBOCHAETE									
			HILOENSIS (954)	20	0	.3	32.4	2	+
			SP (517)	0					+
VOLVOCALES									
CARTERIA									
			CRUCIFERA (432)	3	0	0	36.2	2	+
			EUGAMETOS (2161)	3	0	2.9	28.2	1	+
			EUGAMETOS (233)	3		0.8	-	1	+
CHLAMYDOMONAS									
			ACTINOCHLORIS (965)	0					+
			AGGREGATA (969)	0					0
			AGLOEFORMIS (231)	0					+
			AKINETOS (967)	0					+
			APPLANATA (230)	3	0.1	0.2	0.6	-	-
			CHLAMYDOGAMA (102)	20	0	1.2	14.5	2	-
			DEBARYANA (1344)	20	0.7	13.2	-	1	+
			DEBARYANA (344)	0					+
			DORSOVENTRALIS (228)	3	0	4.4	-	2	-
			ELLIPTICA (1060)	20	0	1.0	24.9	1	+
			ELLIPTICA (1059)	0					0
			EUGAMETOS (10)	3	0.9	3.1	25.9	2	+
			EUGAMETOS (9)	3	1.2	5.0	22.1	2	+
			GLOEOPHILA (607)	0					0
			HINDAKII (1338)	20	0	0	15.1	2	-
			HYDRA (4)	20	0	0.1	0	-	0
			MELANOSPORA (2022)	0					0
			MEXICANA (730)	0					+
			MOEWUSII (2018)	3	0	0	101.1	2	+
			MOEWUSII (2019)	20	0	0	27.0	1	+
			MOEWUSII (96)	3	0	0	26.9	1	+
			MOEWUSII (792)	20	0	0	23.6	10	+
			MOEWUSII (97)	3	3.8	5.8	7.7	-	+
			REINHARDTII (90)	3	0.2	1.0	14.0	5	+

DIVISION ORDER	GENUS SPECIES (UTEX NO) <sup>1</sup>	INDUCTION <sup>2</sup> TIME PRIOR TO ASSAY	DARK <sup>3</sup> RATE	COMP <sup>4</sup> RATE	SAT <sup>5</sup> RATE	% O <sub>2</sub> <sup>6</sup> FOR INHIB	O <sub>2</sub> <sup>7</sup> RATE
	REINHARDTII (89)	20	0	0	1.0	-	+
	REINHARDTII (2246)	20	0	0	0.2	-	+
	SMITHII (1061)	0			-		0
	TEXENSIS (1904)	20	1.0	4.8	-	-	+
	CHLOROGONIUM						
	ELONGATUM (1639)	0					+
	SP (2010)	0					+
	SP (2011)	0					+
	SP (2160)	0					+
	EUDO-PLEO (CROSS)						
	X (1226)	20	0	1.2	35.5	5	+
	X (1225)	20	0	1.2	27.1	2	-
	X (1224)	20	0	1.3	8.8	-	+
	EUDORINA						
	ELEGANS (1193)	20	0.3	4.5	6.0	2	+
	UNICOCCA (737)	0					-
	GONIUM						
	SOCIALE	3	0	0.4	-	2	+
	HAEMATOCOCCUS						
	DROEBAKENSIS (55)	3	0	0.4	-	-	+
	PANDORINA						
	CHARKOWIENSIS (840)	0					+
	MORUM (870)	20	0	0	68.2	5	+
	MORUM (1039)	20	0.5	4.6	27.1	5	+
	MORUM (885)	20	0.1	4.0	19.1	2	+
	MORUM (855)	0					+
	MORUM (876)	0					+
	MORUM (878)	0					+
	MORUM (881)	0					+
	MORUM (1732)	0					+
	UNICOCCA (2031)	0					+
	VOLVOX						
	63H-80	20	0	0.5	13.5	2	+

TABLE II; continued.

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DIVISION	ORDER	GENUS	SPECIES (UTEZ NO)	INDUCTION <sup>2</sup> TIME PRIOR TO ASSAY	DARK <sup>3</sup> RATE	COMP <sup>4</sup> RATE	SAT <sup>5</sup> RATE	% O <sub>2</sub> <sup>6</sup> FOR INHIB	O <sub>2</sub> <sup>7</sup> RATE
		ZYGNEMATALES							
		CLOSTERIUM							
			ACEROSUM (1076)	0					+
		COSMARIUM							
			BOTRYTIS (301)	3	0.3	0.2	-	-	+
			TURPINII (733)	20	0.2	0	-	-	+
		ONYCHONEMA							
			SP (832)	0					0
		ROYA							
			ANGLICA (934)	0					0
		SPIROGYRA							
			PRATENSIS (926)	0					-
		TETRASPORALES							
		PALMELLOPSIS							
			MURALIS (not assigned)	0					0
		TETRASPORA							
			SP (234)	0					0
		ULOTRICHALES							
		RADIOFILUM							
			TRANSVERSALE (1252)	0					+
		STICHOCOCCUS							
			BACILLARIS (176)	0					+
		ULOTHRIX							
			GIGAS (174)	3	0.1	1.0	34.9	1	+
		CHRYSOPHYCOPHYTA							
		AULOSIRA							
			TERRESTERE (1824)	0					-
		BUMILLERIA							
			SICULA (172)	0					+
		BUMILLERIOPSIS							
			PETERSENIANA (147)	0					+
		PLEUROCHLORIS							
			COMMUTATA (310)	0					+
		PSEUDOBUMILLERIOPSIS							
			PYRENOIDOSA (980)	0					+

DIVISION ORDER	GENUS SPECIES (UTEX NO) <sup>1</sup>	INDUCTION <sup>2</sup> TIME PRIOR TO ASSAY	DARK <sup>3</sup> RATE	COMP <sup>4</sup> RATE	SAT <sup>5</sup> RATE	% O <sub>2</sub> <sup>6</sup> FOR INHIB	O <sub>2</sub> <sup>7</sup> RATE
RHODOPHYCOPHYTA	GLAUCOSPHAERA VACUOLATA (1662)	0					-
	PORPHYRIDIUM AERUGINEUM (755)	0					+
CRYPTOPHYCOPHYTA	CRYPTOMONAS OVATA (358)	3	0.8	0.8	-	-	0
EUGLENOPHYCOPHYTA	EUGLENA GRACILIS (884)	0					+
CYANOPHYCOPHYTA	MICROCYSTIS AERUGINOSA (2061)	3	2.0	0	0		+

All rates are expressed as initial  $\mu$  moles H<sub>2</sub> evolved (mg chlorophyll)<sup>-1</sup> hr<sup>-1</sup>. Dashes indicate missing data points.

1. UTEX numbers refer to strain numbers from the Culture Collection of Algae at the University of Texas at Austin.
2. Induction times (3 or 20 hr) were selected which gave highest saturating rate of H<sub>2</sub> production for the algal strain. A zero indicates no H<sub>2</sub> production at 3 or 20 hr.
3. Dark rate was recorded during the first (approximately) three minutes subsequent to transferring algae to the assay chamber.
4. Compensating rate was recorded for the few minutes immediately after dim light exposure was begun, such that no net O<sub>2</sub> evolution occurred.
5. Saturating rate was the maximum attained under saturating light intensity.
6. Numbers refer to the % of air-saturating O<sub>2</sub> in solution required to inhibit the saturating rate by approximately 50%.
7. Plus indicates cells were capable of O<sub>2</sub> evolution in presence of saturating light subsequent to induction. Zero indicates no O<sub>2</sub> evolution observed at any time subsequent to the induction treatment. Dash indicates missing datum point.

TABLE III  
GREEN ALGAE TESTED FOR  
HYDROGENASE ACTIVITY

ORDER	NUMBER OF STRAINS TESTED	15% GENERATING H <sub>2</sub>
Chlorellales	18	44
Oedogoniales	2	50
Tetrasporales	2	0
Ulotrichales	3	33
Volvocales	52	67

TABLE IV  
VOLVOCALES TESTED FOR  
HYDROGENASE ACTIVITY

GENUS	NUMBER STRAINS TESTED	15% GENERATING H <sub>2</sub>
Carteria	3	100
Chlamydomonas	28	71
Chlorogonium	4	25
Eudo-Pleo	3	100
Eudorina	2	100
Gonium	1	+
Haematococcus	1	+
Pandorina	10	40
Volvox	1	+

TABLE V  
 MAXIMUM RATE OF HYDROGEN PRODUCTION  
 WITHIN CHLAMYDOMONAS

Species	Culture Collection Number	Rate [ $\mu\text{moles (mg Chl)}^{-1} \text{ hr}^{-1}$ ]
moewusii	2018	101.12
moewusii	2019	27.03
moewusii	96	26.91
eugametos	10	25.90
elliptica	1060	24.90
moewusii	792	23.60
eugametos	9	22.05
hindakii	1338	15.06
chlamydogama	102	14.50
reinhardtii	90	14.00
debaryana	1344	13.22
texensis	1904	7.80
moewusii	97	7.70
dorsoventralis	228	4.42
reinhardtii	89	1.44
applanata	230	0.62
reinhardtii	2246	0.27
hydra	4	0.11
agloeformis	231	0.00
debaryana	344	0.00
gloeophila	607	0.00
mexicana	730	0.00
actinochloris	965	0.00
akinetos	967	0.00
aggregata	969	0.00
elliptica	1059	0.00
smithii	1061	0.00
melanospora	2022	0.00

Rates are all expressed as initial velocity of  $\text{H}_2$  photoproduction in saturating light. Induction times were 3 or 20 hr, depending on which gave highest rate for each variety.

indicate that others listed below C. hydra in Table V also have hydrogenase activity, but the level of activity is below our limits of detection. The time limit imposed for completion of this work precluded more detailed analysis of most strains but recent experiments with C. eugametos (UTEX 10) have shown that culture conditions prior to induction considerably influence the rate of H<sub>2</sub> production, suggesting further that some strains which demonstrated no hydrogenase activity might do so under different conditions. Finally, since many strains had significant H<sub>2</sub> evolution at 20 hr induction but no activity at 3 hr induction it seems likely that a few would begin generating H<sub>2</sub> at longer induction times. Therefore, the actual number of algal strains capable of photoproduction of H<sub>2</sub> among those examined may be higher than that reported here, and we regard the numbers presented in Tables III-V as minima.

One strain of C. elliptica (UTEX 1060) had a high rate of H<sub>2</sub> photoproduction while strain UTEX 1059, which is its mating partner of opposite strain, had no detectable activity. This result was surprising, although mating pairs among Chlamydomonas often occur between morphologically distinct strains, suggesting significant biochemical differences may also occur between these strains.

In our efforts to identify strains with "best" hydrogenase activity any of several criteria could be used to define best. If highest maximum rate is sought then Chlamydomonas moewusii (UTEX 2018) is clearly best, with Pandorina morum (UTEX 878) second (Table II). A number of other active strains had rates of over 20  $\mu$  moles H<sub>2</sub> (mg chlorophyll)<sup>-1</sup> hr<sup>-1</sup>, with strains of C. moewusii/elliptica generally having highest activity.

Strains with "best" hydrogenase activity might be defined as those with activity least sensitive to dissolved O<sub>2</sub> in solution. Of those tested, virtually all lost most hydrogenase activity when 2% of the air-saturating amount of O<sub>2</sub> was introduced. Several strains retained significant activity after 5% O<sub>2</sub> was introduced, and Pandorina morum (UTEX 870) retained over 50% of its initial activity in the presence of 10% of air saturating O<sub>2</sub>. These results are hard to interpret, however, since the oxy-hydrogen reaction begins immediately upon addition of O<sub>2</sub> and we did not illuminate the sample until this O<sub>2</sub>-uptake reaction was complete. Thus, our assays measured the extent to which hydrogenase activity was destroyed during the 1 to 5 minutes in O<sub>2</sub> prior to completion of the oxy-hydrogen reaction. It did not measure the steady-state rate of H<sub>2</sub> photoproduction in the presence of a constant amount O<sub>2</sub> in solution. This latter kind of experiment would be hard to perform in a small closed chamber as used in these experiments.

A third way of defining "best" hydrogen producing capacity is those which generate H<sub>2</sub> after a very short induction. Our experiments were not designed to detect hydrogenase activity in cells induced for less than three hr. By accident we noted that Chlamydomonas eugametos (UTEX 10) began producing H<sub>2</sub> subsequent to less than 10 min. induction time. Recent experiments were performed in which C. eugametos was induced directly in the assay chamber. H<sub>2</sub> evolution began even in darkness, when induction time was only 4 minutes, to our knowledge a much shorter induction than any previously reported.

The data were examined for other correlations and unusual observations. When rates of H<sub>2</sub> production in the dark and in the light were compared most strains had rates at least an order of magnitude higher under saturating light. However, some strains had a substantial rate of dark photoproduction, which was not increased

TABLE VI  
SPEARMAN CORRELATION COEFFICIENTS  
FOR VARIOUS ASSAY PARAMETERS

SPEARMAN CORRELATION COEFFICIENTS / PROB > |R| UNDER  $H_0: \rho=0$  / NUMBER OF OBSERVATIONS

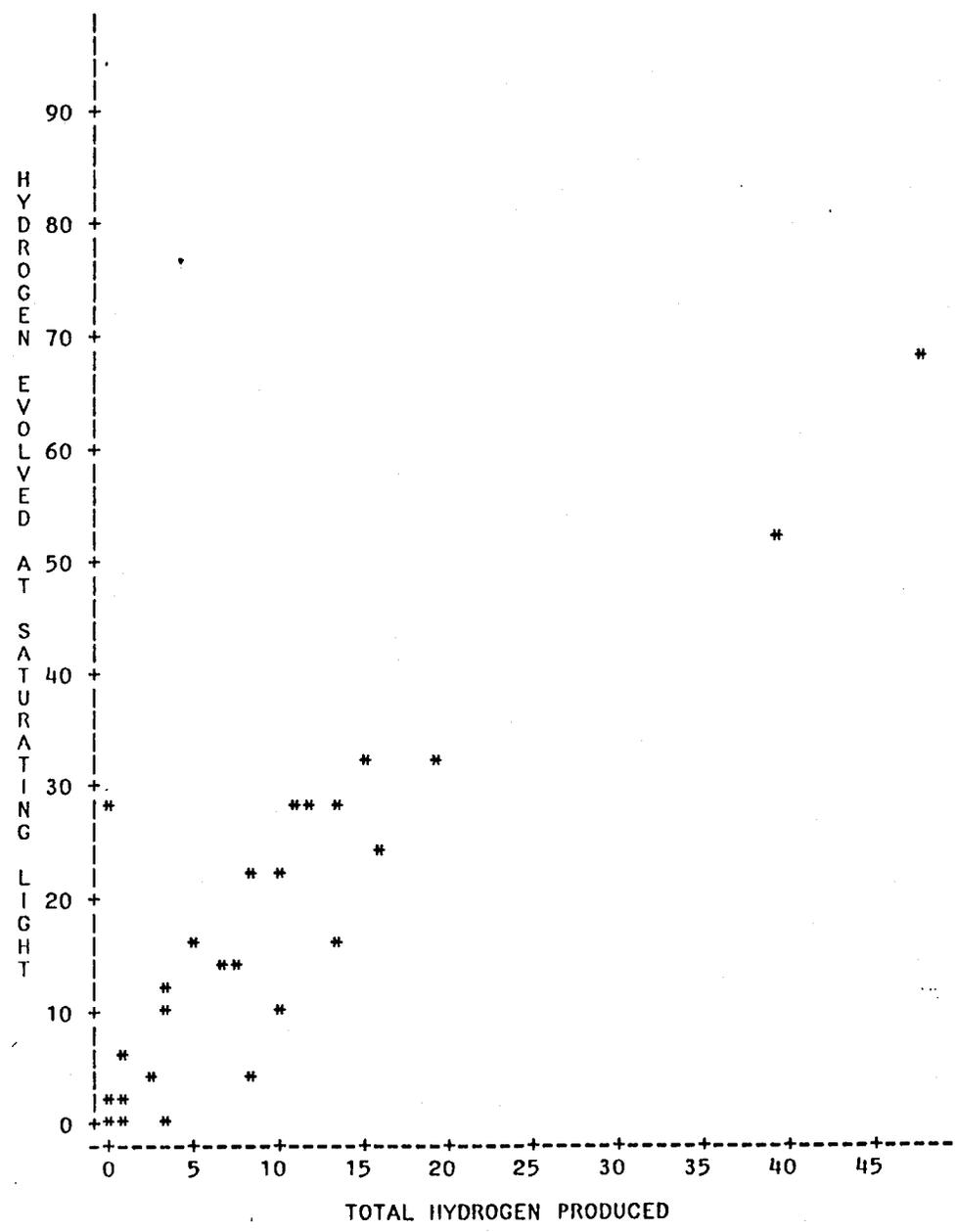
	COMP300	SAT300	TOT300	COMP200	SAT200	TOT200	CHLORO
COMP300	1.00000	0.77474	0.79284	0.63858	0.67364	0.67279	0.06431
H2 PRODUCED AT COMP LIGHT ( 3 HRS)	0.0000 117	0.0001 76	0.0001 107	0.0001 113	0.0001 74	0.0001 92	0.5084 108
SAT300	0.77474	1.00000	0.97773	0.52935	0.85998	0.86818	0.16678
H2 EVOLVED AT SAT LIGHT ( 3 HRS)	0.0001 76	0.0000 76	0.0001 72	0.0001 73	0.0001 71	0.0001 62	0.1676 70
TOT300	0.79284	0.97773	1.00000	0.57484	0.87075	0.90823	0.04611
TOTAL H2 PRODUCED ( 3 HRS)	0.0001 107	0.0001 72	0.0000 107	0.0001 104	0.0001 70	0.0001 90	0.6504 99
COMP200	0.63858	0.52935	0.57484	1.00000	0.65454	0.64472	0.06532
H2 PRODUCED AT COMP LIGHT (20 HRS)	0.0001 113	0.0001 73	0.0001 104	0.0000 116	0.0001 76	0.0001 93	0.5039 107
SAT200	0.67364	0.85998	0.87075	0.65454	1.00000	0.93937	0.09087
H2 EVOLVED AT SAT LIGHT (20 HRS)	0.0001 74	0.0001 71	0.0001 70	0.0001 76	0.0000 76	0.0001 64	0.4544 70
TOT200	0.67279	0.86818	0.90823	0.64472	0.93937	1.00000	0.06427
TOTAL H2 PRODUCED (20 HRS)	0.0001 92	0.0001 62	0.0001 90	0.0001 93	0.0001 64	0.0000 93	0.5590 85
CHLORO	0.06431	0.16678	0.04611	0.06532	0.09087	0.06427	1.00000
CHLOROPHYLL CONCENTRATION ( G/ML)	0.5084 108	0.1676 70	0.6504 99	0.5039 107	0.4544 70	0.5590 85	0.0000 111

TABLE VII  
PEARSON CORRELATION COEFFICIENTS  
FOR VARIOUS ASSAY PARAMETERS

CORRELATION COEFFICIENTS / PROB > |R| UNDER H<sub>0</sub>:RHO=0 / NUMBER OF OBSERVATIONS

	COMP300	SAT300	TOT300	COMP200	SAT200	TOT200	CHLORO
COMP300	1.00000	0.34525	0.26469	0.46745	0.36084	0.21983	-0.00191
H2 PRODUCED AT COMP LIGHT ( 3 HRS)	0.0000 117	0.0023 76	0.0059 107	0.0001 113	0.0016 74	0.0352 92	0.9843 108
SAT300	0.34525	1.00000	0.86976	0.10219	0.24884	0.22274	0.11793
H2 EVOLVED AT SAT LIGHT ( 3 HRS)	0.0023 76	0.0000 76	0.0001 72	0.3896 73	0.0364 71	0.0818 62	0.3309 70
TOT300	0.26469	0.86976	1.00000	-0.02364	0.18240	0.29709	-0.04759
TOTAL H2 PRODUCED ( 3 HRS)	0.0059 107	0.0001 72	0.0000 107	0.8117 104	0.1307 70	0.0045 90	0.6400 99
COMP200	0.46745	0.10219	-0.02364	1.00000	0.38778	0.07516	0.01952
H2 PRODUCED AT COMP LIGHT (20 HRS)	0.0001 113	0.3896 73	0.8117 104	0.0000 116	0.0005 76	0.4740 93	0.8418 107
SAT200	0.36084	0.24884	0.18240	0.38778	1.00000	0.92773	0.12101
H2 EVOLVED AT SAT LIGHT (20 HRS)	0.0016 74	0.0364 71	0.1307 70	0.0005 76	0.0000 76	0.0001 64	0.3183 70
TOT200	0.21983	0.22274	0.29709	0.07516	0.92773	1.00000	0.01664
TOTAL H2 PRODUCED (20 HRS)	0.0352 92	0.0818 62	0.0045 90	0.4740 93	0.0001 64	0.0000 93	0.8799 85
CHLORO	-0.00191	0.11793	-0.04759	0.01952	0.12101	0.01664	1.00000
CHLOROPHYLL CONCENTRATION ( G/ML)	0.9843 108	0.3309 70	0.6400 99	0.8418 107	0.3183 70	0.8799 85	0.0000 111

RATE OF PRODUCTION VS. TOTAL AMOUNT PRODUCED  
HYDROGEN AT TWENTY HOURS



NOTE: 56 OBS HAD MISSING VALUES 38 OBS HIDDEN

under compensating light. Chlamydomonas moewusii (UTEX 97) had a dark rate of  $3.7 \mu \text{ moles H}_2 (\text{mg chlorophyll})^{-1} \text{ hr}^{-1}$  which was increased only to 7.7 in saturating light. At the other extreme several strains had no detectable  $\text{H}_2$  dark, but had high rates at saturating light intensity.

In order to examine the data for possible correlations between assay parameters correlation coefficients were determined (Tables VI and VII). Pearson coefficients, a measure of linear correlation, were highest for the correlations between saturating rate of  $\text{H}_2$  production and total  $\text{H}_2$  produced for a given induction time. The coefficients were 0.87 and 0.93 for 3 and 20 hr, respectively - remarkably high values considering the diverse nature of the data set. Spearman correlations were tested for significance instead of Pearson's, due to the possible nonnormality of the data. The Spearman coefficients for this relationship were 0.98 and 0.94 respectively, and significant at the  $<0.0001$  level. Since there was only a slight increase in the coefficients in going from Pearson's to Spearman's, a linear relationship is implied. Other correlations in Tables VI and VII were statistically significant, but due to their lower coefficients, were deemed biologically insignificant.

The relationship between total hydrogen produced and saturating rate of  $\text{H}_2$  production in cells induced for 20 hr is shown in Fig. 1. The general linear nature of the curve is evident. The same plot for cells induced for 3 hr appeared very similar. When other pairs of variable sets were plotted much more scatter appeared in the data and linear relationships were not evident.

#### CONCLUSIONS AND FUTURE DIRECTIONS

It was hoped that clear taxonomic patterns would be demonstrated from this study. Trends were evident, with green algae predominantly having hydrogenase, especially Volvocales and Chlorococcales. Yet, within a single genus (Ankistrodesmus, Chlamydomonas or Chlorella, for example) some species demonstrated hydrogenase activity while others did not. Even within the same species (in Chlamydomonas elliptica and in Pandorina morum) one strain evolved  $\text{H}_2$  while another did not. This could result from very low rates in strains of which we failed to detect  $\text{H}_2$  production or could result from not choosing appropriate conditions for them to generate  $\text{H}_2$ . These possibilities could be tested by examining closely related varieties at higher sensitivity and under a more broad range of conditions in which some evolve  $\text{H}_2$  and others seemingly do not. However, it is also possible that the hydrogenase enzyme is easily evolved from some pre-existing, very similar protein. Those species or strains which often find themselves temporarily in anaerobic circumstances, which might be expected in soil algae as many Volvocales are, might evolve a hydrogenase enzyme to remove excess reductant during fermentation. It is interesting that some strains with no hydrogenase activity also demonstrated no photosynthetic  $\text{O}_2$  evolution subsequent to induction, suggesting that the reducing environment was toxic to them. In a few strains we observed nearly complete loss of  $\text{O}_2$ -evolving capacity after only a few minutes of anaerobic incubation, and these strains never produced any  $\text{H}_2$ . Although a large number of green algal species have been isolated from soil and studied in culture, very few studies have concentrated on the ecological patterns and environmental conditions of individual cells in their natural soil habitat. Perhaps the most definitive work is still the monograph by J. P. Peterson, "Studies on the Biology

and Taxonomy of Soil Algae", written in 1935. It would be interesting to determine if a correlation between  $H_2$ -evolving capacity and ability to survive under the immediate surface of the soil exists.

In attempting to identify eukaryotic algae which might be most useful for future studies of hydrogenase activities, several interesting strains emerged. The very high initial rate of  $H_2$  photoproduction in *Chlamydomonas moewusii* (UTEX 2018) may not reflect a particularly active hydrogenase system (see below) and we do not propose it as a strain especially suited for further investigation. *C. eugametos* (UTEX 10) does appear very interesting because healthy cultures of it can be induced to start generating  $H_2$  within 4 minutes. This is a very short time for genetic induction of an enzyme, which suggests that it may simply be in some way activated by a reducing environment. We have recently observed that cyclohexamide or chloramphenicol in concentrations which stop steady-state growth do not prevent induction of hydrogenase within a few minutes in this strain.

Other strains of particular interest include those which are able to withstand temporary exposure to relatively high concentrations of  $O_2$  without losing hydrogenase activity. This may not indicate a hydrogenase with particularly high  $O_2$  tolerance, but instead one which is inaccessible enough that the excess  $O_2$  is used up in the oxy-hydrogen reaction or in some other respiratory process before it has time to contact and inactivate the hydrogenase enzyme. Thus, cells with relatively impermeable cell walls and colonial types with an external matrix might be especially resistant under our experimental conditions. These proposals can be tested by removing  $H_2$  from the culture prior to introducing  $O_2$  (to eliminate the oxy-hydrogen reaction) and by adding respiratory inhibitors. If the hydrogenase is in fact less sensitive to  $O_2$  in some strains, then it should be possible to successfully induce them in the presence of some  $O_2$  in solution.

In every strain we examined the initial rate of  $H_2$  photoproduction was sustained only briefly, then ceased entirely. Under saturating light this can easily be explained as poisoning of the enzyme by  $O_2$  generated in photosynthesis. However, the compensating light intensity generated no net  $O_2$ . Yet  $H_2$  production ceased after a few minutes even in this dim light and generally less total  $H_2$  was produced in compensating light than under saturation. It is possible that internal  $O_2$  generated in dim light is enough to poison the hydrogenase. It seems more likely, however, that  $H_2$  production (even photoproduction) depends on some internal pool of reductant, not just photolysis of water. This proposal has been forwarded by many ever since Gaffron observed an effect of externally added glucose on hydrogenase activity. Perhaps our most interesting observations bearing on this proposal are those presented in Tables VI and VII, and in Fig. 1. These data strongly suggest a direct linear relationship between initial velocity of  $H_2$  photoproduction and total amount of  $H_2$  generated in saturating light before the reaction ceased. If the reaction were limited by the hydrogenase enzyme concentration or its specific activity, then there is no reason why this correlation should exist. However, suppose that  $H_2$  photoproduction is limited by some substrate required for the reaction. Assuming a single substrate Michaelis-type reaction with non-renewable substrate used up during the reaction, a linear relationship between initial velocity and total amount evolved would be expected. The shapes of the curves of rate of  $H_2$  photoproduction vs. time are as would be predicted for this relationship. The purpose of a depletable substrate is not clear since DCMU experiments suggest that some reducing equivalents transferred to  $H^+$  do in fact

come from water, and  $O_2$  is often observed to be evolved stoichiometrically with  $H_2$  photoproduction. Perhaps, as has been proposed by others, the substrate is required to generate ATP which is in some way required in the overall process. However, the relationship between photosynthesis and other metabolic processes which occur during  $H_2$  photoproduction are still poorly understood.

We conclude from this study that  $H_2$  photoproduction may not be possible in unmodified eukaryotic algae under conditions whereby  $H_2$  could be collected as an economically feasible energy source. This does not preclude the possibility that strains which already have an active hydrogenase system could be altered genetically to improve the feasibility of commercial  $H_2$  photoproduction. At present it is impossible to assess even the possibility of steady-state  $H_2$  photoproduction in eukaryotic algae because we do not know enough about the underlying biochemistry to propose a specific approach to the problem. It would probably be worthwhile to extend our survey somewhat in order to examine in more detail taxonomic groups which show the most promise and to examine others which, to date, have not been tested for hydrogenase activity. However, the most important approach would be to examine in detail metabolic processes associated with  $H_2$  photoproduction. This is currently being done in several laboratories, including those of SERI, but the complexity and importance of the problem warrants more intensive research in a more broad spectrum of laboratories.