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WHOLE CELL AND CELL-FREE HYDROGENASES OF ALGAE*

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Abstract—For the first time several species of algae are found to exhibit hydrogenase activity after dark adaptation. Seven of the species examined do not exhibit hydrogenase activity even after 48 hr of attempted adaptation. In whole cells of *Chlamydomonas moewusii*, the pH optimum of the adaptation process is 7·5. The pH optimum of the enzyme is 9·7 after adaptation. In another *Chlamydomonas* species, the pH optimum of the enzyme for a cell-free extract from adapted whole cells is 7·0. The hydrogenases of algae appear to have isoenzyme patterns and substrate specificities similar to those of bacterial enzymes. The adaptation process proceeds in the presence of carbon monoxide which inactivates the enzyme. This indicates that the appearance of hydrogenase activity may be controlled by the level of some metabolite(s) not immediately connected with the hydrogenase reaction. Inhibition of hydrogenase activity by carbon monoxide is reversed by removing the gas. Hydrogenase from dried cell preparations of *C. moewusii* and *Chlamydomonas* sp. is more stable to air than the enzyme obtained from whole cell suspensions of the same organisms.

INTRODUCTION

HYDROGENASES play an important role in the anaerobic metabolism of many species of bacteria.[1] Present knowledge concerning such enzymes is based upon studies of hydrogenase preparations extracted from bacterial cells. Similar work with hydrogenases of algae has lagged. This may be due in part to the low level of hydrogenase activity and the sensitivity of the enzymes to oxygen. Also, some kinds of algal cells are difficult to rupture without destroying enzyme activity. Since Gaffron's discovery of adaptive hydrogenase in *Scenedesmus*,[2] algal species from five taxonomic divisions have been shown to exhibit hydrogenase activity.[3] The process of adapting algal cells to exhibit hydrogenase activity requires from several minutes to days.[2, 4, 5] Under conditions of adaptation the algae are moribund and bacterial contamination as a significant source of hydrogenase is a constant concern.

This report deals with some of the physical properties and adaptation processes of several algal hydrogenases obtained in dried cell suspensions and cell-free extracts. A comparison of some of these properties with those of whole cell suspensions is also reported.

RESULTS AND DISCUSSION

Three different types of adaptation time courses (Fig. 1) are exhibited by the various algae studied. Type I exemplifies the kind of curve obtained from *Chlamydomonas moewusii* and other *Chlamydomonas* sp. Initial adaptation time is short (10 min). Maximum

* This paper is part of a dissertation submitted to the Graduate Division of the University of Hawaii in partial fulfillment of the requirements for the Ph.D. degree.

[1] C. T. GRAY and H. GEST, *Science* **149**, 186 (1965).
[2] H. GAFFRON, *Am. J. Botany* **27**, 273 (1940).
[3] C. J. P. SPRUIT, *Physiology and Biochemistry of Algae*, p. 49, Academic Press, New York (1963).
[4] A. W. FRANKEL and R. A. LEWIN, *Am. J. Botany* **41**, 586 (1954).
[5] K. DAMASCHKE, *Z. Naturforsch.* **12b**, 441 (1957).

adaptation is reached at approximately 18 hr and is followed by a steady decrease in hydrogenase activity after that time. Type II is similar in shape but differs in one aspect. Initial adaptation is not reached until 20 hr. In Type III, adaptation does not reach a maximum even after 72 hr dark adaptation. The decrease in hydrogenase activity after 18 hr in the Type I curve is presumably due to progressive destruction of the cells and enzymatic destruction of hydrogenase. This decrease agrees with the previous observations of Kessler using *Ankistrodesmus*,[6] *Selenastrum* (time of maximum hydrogenase activity, 4 hr), *Coelastrum* (time of maximum hydrogenase activity, 4 hr),[7] and a variety of *Chlorella* species and strains.[8] The time of maximum adaptation and level of hydrogenase production can vary widely as seen by the present work with various *Chlamydomonas* species and by the work of Kessler with species of *Chlorella*.[8]

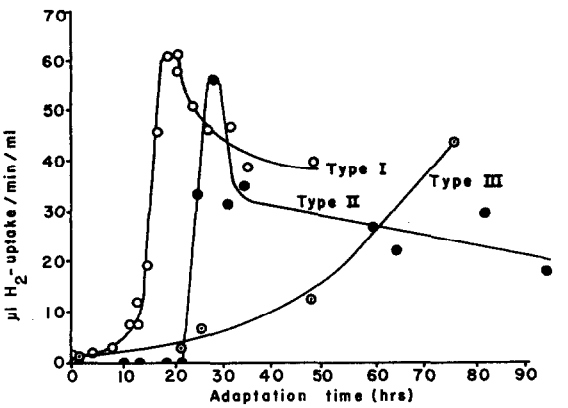

FIG. 1. TYPES OF ADAPTATION TIME CURVES FOR WHOLE CELLS OF ALGAE.

Hydrogenase activity for each time period was measured by manometric assay following H_2-uptake with methylene blue. One side-arm of the flask contained 6·4 µmoles of methylene blue in 0·2 ml of 0·1 M phosphate buffer (pH 7·0): the other contained 0·5 g (wet wt.) of adapted whole cells suspended in 0·5 ml buffer. The center well contained 0·1 ml of 14% NaOH into which was added 0·1 ml of 20% pyrogallol just prior to gassing. The total volume was made to 3·2 ml with phosphate buffer. The gas phase was hydrogen. A control (without dye) was run with each assay.

○——○: Time course curve obtained with *Chlamydomonas moewusii*.
●——●: Time course curve obtained with *Chlamydomonas eugametos* Strain No. 4.
⊙——⊙: Time course curve obtained with *Chlamydomonas eugametos* Strain No. 6.

Table 1 shows the time of maximum adaptation of hydrogenase activity for various algae, level of hydrogenase activity at this time for whole cells, the time at which initial activity is detected, the level of activity at this time, and the type of adaptation profile exhibited by the algal species (see Fig. 1). The hydrogenase activities of cell-free preparations from three of the algae (Table 1) are expressed as per cent of activity present in the whole cells. Active cell-free preparations from adapted *Chlorella pyrenoidosa* could not be obtained by the method described, by use of a French Pressure Cell, or by sonication prior to drying the suspension. *Chlamydomonas intermedia* cells are surrounded by a thick, gelatinous matrix,

[6] E. KESSLER, *Handb. Pflanzenphysiol.* 5, 951 (1960).
[7] E. KESSLER and H. MAIFARTH, *Arch. Mikrobiol.* 37, 215 (1960).
[8] E. KESSLER, W. LANGNER, I. LUDEWIG and H. WIECHMANN, *Studies on Microalgae and Photosynthetic Bacteria*, p. 7, University of Tokyo Press, Tokyo (1963).

making cell-free extraction impossible. Efforts to free the cells from the matrix by the use of detergents destroyed hydrogenase activity. The low hydrogenase activities measured in these cells result from the fact that a major portion of the dry weight of the alga is contributed by the gelatinous matrix. Efforts to obtain cell-free preparations from the strains of *Chlamydomonas eugametos* were not made due to the unusually long time required for the cells to reach maximum adaptation. *Nostoc muscorum*, previously reported as not having hydrogenase activity,[6] exhibited an activity of 5·4 μl hydrogen evolved/min/g wet wt. after 21 hr adaptation when reduced methyl viologen was used as the electron donor. *Chlamydomonas pseudogloe*, *Gloeocapsa* sp., and species of *Caulerpa*, *Dictyosphaeria*, *Hypnea*, *Sarggassum*, and *Turbinaria* do not exhibit hydrogenase activity after 48 hr under the conditions described. From Table 1 the algae most suitable for further investigation are *Chlamydomonas moewusii* and *Chlamydomonas* sp. since adaptation times are short, and activities are relatively high. Also, active hydrogenase can be easily extracted from these cells.

TABLE 1. HYDROGENASE ADAPTATION CHARACTERISTICS OF VARIOUS ALGAL SPECIES

Organism	Initial adaptation		Maximum adaptation		Adaptation type*	Cell-free activity (% of whole cells)
	Time (hr)	Activity†	Time (hr)	Activity		
Chlamydomonas sp.	0·16	3·1	29·0	68·7	I	34·0
Chlamydomonas moewusii	0·20	1·3	21·0	61·4	I	27·7
Chlamydomonas intermedia	19·5	2·3	44·0	11·1	II	—
Chlamydomonas eugametos No. 4	22–25	33·0	28·0	57·0	II	—
Chlamydomonas eugametos No. 5	24·0	1·5	234·0	112·0	III	—
Chlamydomonas eugametos No. 6	21·5	2·75	166·0	90·0	III	—
Scenedesmus obliquus	0·25	1·5	44·0	24·5	I	22·3
Chlorella pyrenoidosa	20·0	18·0	21·0	29·6	II	—
Ulva faciata	17–21	1·76	21·0	1·76	II	—

* See Fig. 1.
† Activities expressed as μl. H_2 uptake/min/g wet wt.

Hydrogenase production as a function the pH of the adaptation medium is shown in Fig. 2. A pH profile of hydrogenase after adaptation is also shown. Both curves represent whole cells of *Chlamydomonas moewusii*. The pH optimum is 7·0 for cell-free preparations of *Chlamydomonas* sp. (4·0 μl H_2/min/0·1 g dry wt.), although the total activity of the preparation is considerably lower than the whole cells. It is different from that obtained from whole cells after adaptation. The pH curve obtained with whole cells of *Chlamydomonas moewusii* after adaptation is similar to that obtained by Abeles from a cell-free preparation of *Chlamydomonas eugametos*,[9] following hydrogen uptake via methylene blue reduction. It is different from the optimum of 8·5 obtained by Sadana and Jagannathan[10] for cell-free preparations from *Desulfovibrio desulfuricans* using methylene blue, and the optimum of 5·5 obtained by Krasna *et al.*[11] following the hydrogen exchange reaction with deuterium oxide

[9] F. B. ABELES, *Plant Physiol.* 39, 169 (1964).
[10] J. C. SADANA and V. JAGANNATHAN, *Biochem. Biophys. Acta* 19, 440 (1956).
[11] A. I. KRASNA, E. RILIS and D. RITTENBERG, *J. Biol. Chem.* 235, 2717 (1960).

with purified extracts of *Desulfovibrio desulfuricans* hydrogenase. The pH optimum of 6·8 obtained by Asato[12] from cell-free extracts of *Clostridium butylicum*, utilizing the methylene blue reaction, is similar to the optimum obtained here for cell-free extracts of *Chlamydomonas* sp.

Whole cells of *Chlamydomonas* sp. were adapted under hydrogen, nitrogen, and carbon monoxide for periods of 2, 4 and 7 hr. Identical levels of hydrogenase activity were obtained for cells adapted under hydrogen and nitrogen at each time period as measured with reduced

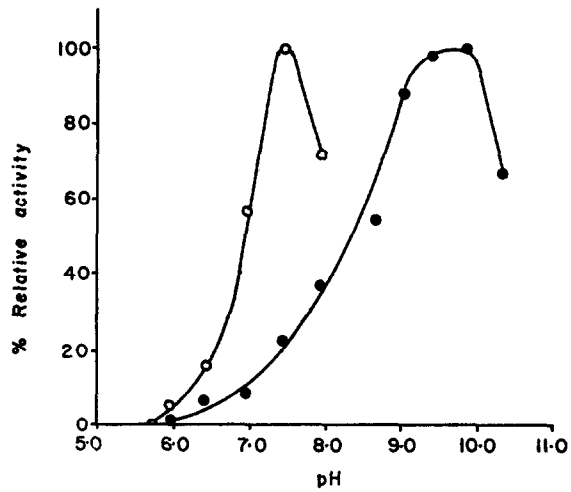

FIG. 2. pH PROFILE OF HYDROGENASE ACTIVITY FROM WHOLE CELLS OF *Chlamydomonas moewusii* ADAPTED AT pH 7·0 AND pH PROFILE OF THE ADAPTATION PROCESS ITSELF.

Hydrogenase assays of cells adapted at pH 7·0 were as under Fig. 1 except that 0·1 ml of the algal suspension was used. 0·1 M phosphate buffers ranging in pH from 5·7 to 8·0 and 0·1 M glycine–NaOH buffers ranging in pH from 8·6 to 10·34 were used. The adaptation time was 22 hr. The pH profile of the adaptation as under Fig. 1 except that 0·1 ml of the algal suspension was used. The adaptation time was 22 hr 0·1 M phosphate buffers ranging in pH from 5·7 to 8·0 and 0·1 M glycine–NaOH buffers ranging in pH from 8·6 to 10·34 were used. The pH profile of the adaptation process was done by suspending the whole cells in 0·1 M phosphate buffers (0·5 g wet wt./ml) over pH range 5·7–8·0. Assays were carried out at pH 7·0. The adaptation time for this experiment was 24 hr. Flasks minus methylene blue were run with each assay as controls. The two experiments were carried out with two different cultures of cells of *Chlamydomonas moewusii*. The gas phase was H_2.

●——●: pH profile of whole cells adapted at pH 7·0.
○——○: pH profile of whole cells adapted at various pH's and assayed at pH 7·0.

methyl viologen, and with helium as the gas phase. Cells adapted under carbon monoxide exhibit an activity 50 per cent of that obtained with the other gases. Carbon monoxide is a strong inhibitor of bacterial hydrogenases, and in some instances the inhibition can be reversed by either removing the carbon monoxide or by light.[13] Figure 3 shows that carbon monoxide is also an inhibitor of hydrogenase in *Chlamydomonas* sp., and that the inhibition is reversed by removing the gas. Figure 3 also shows that no hydrogen is evolved while carbon monoxide is present. After the Warburg flasks are flushed with helium, the hydrogenase activity is restored to 20 per cent of its original activity. This relatively low activity is probably due to incomplete removal of carbon monoxide.

[12] R. N. ASATO, Ph.D. Dissertation, University of Hawaii, p. 110 (1968).
[13] L. PUREC, A. I. KRASNA and D. RITTENBERG, *Biochem.* 1, 270 (1962).

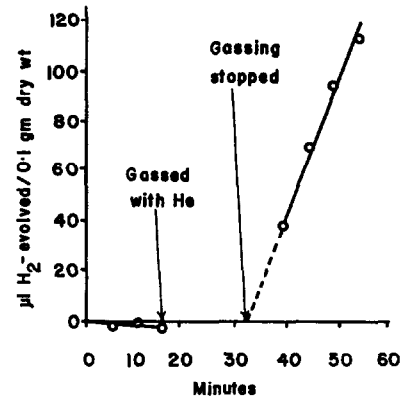

FIG. 3. REVERSIBLE INHIBITION OF HYDROGENASE ACTIVITY WITH CARBON MONOXIDE.

A whole cell suspension (0·6 g wet wt./ml) and assayed for hydrogenase activity via H_2 evolution with reduced methyl viologen under He. 5·5 µmoles of methyl viologen were reduced with 3 µmoles of sodium dithionite and used in the assay. The activity obtained was 28 µl/min/0·1 g dry wt. The assay was then repeated using CO in the gas phase. 16 min after tipping, the Warburg flasks were flushed with He for 15 min and the reaction allowed to proceed. Identical flasks without reduced methyl viologen served as controls.

The fact that the adaptation process can take place under conditions where any hydrogenase activity is inhibited by carbon monoxide suggests that this enzyme does not participate in its own activation. Furthermore, this result indicates that even an indirect autocatalytic mechanism[10, 14] of adaptation is not operating in this case.

Adaptation also occurs in the presence of millimolar quantities of AsO_4^{---}, F^-, and HSO_3^-. It does not, however, occur in the presence of 10^{-3} M iodoacetate.

Hydrogenase activity from dried cell suspensions of *Chlamydomonas moewusii* and *Chlamydomonas* sp. varies in the relative rate of hydrogen uptake with various hydrogen acceptors and in the rate of hydrogen evolution from reduced methyl viologen (Table 2). These results are in general agreement with the work of Abeles,[9] who examined the reaction of some of these hydrogenase acceptors with cell-free preparations of *Chlamydomonas*

TABLE 2. RECEPTOR SPECIFICITY FOR HYDROGENASE FROM DRIED CELL PREPARATIONS OF ADAPTED WHOLE CELLS

Redox dye	E_0	Hydrogenase activity (µl H_2 uptake/min/0·1 g dry wt.)	
		Chlamydomonas moewusii	*Chlamydomonas* sp.
Methylene blue	+0·01 V	5·95	7·55
Benzyl viologen	−0·357 V	5·15	6·13
Methyl viologen	−0·447 V	1·15	2·11
1-1′-Trimethylene-2-2′-dipyridilium-diiodide	−0·56 V	0·40	0·00
1-1′-Tetramethylene-2-2′-bipyridylium-diiodide	−0·64 V	0·00	0·00
1-1′-Trimethylene-5-5′-dimethyl-2-2′-dipyridylium dibromide	−0·64 V	0·00	—
Reduced methyl viologen	−0·447 V	−2·27	—

[14] H. HARTMAN and A. I. KRASNA, *J. Biol. Chem.* **238**, 749 (1963).

eugametos. The acceptor specificity is characteristic of facultative, aerobic, and photosynthetic bacteria whose hydrogenases, in contrast to hydrogenases of anaerobic bacteria, react faster in reduction of methylene blue than in oxidation of reduced methyl viologen. Further, the rate of substrate reduction can be correlated with the oxidation–reduction potential of the substrate. The redox dyes of more positive potential, in general, undergo reduction at a rate faster than those of more negative potential. This result is in agreement with the studies of Asato (unpublished observations), who studied the hydrogenases of bacteria.

The isoenzyme band patterns for three algae hydrogenases are depicted in Fig. 4. The low activity and general sensitivity to oxygen make the detection of these isoenzymes more difficult than with the bacterial hydrogenases. However, with the more stable algal hydrogenases it is possible to establish with certainty the existence of several hydrogenase species within a given algal preparation. As with the bacteria,[15] these have reproducible R_f values

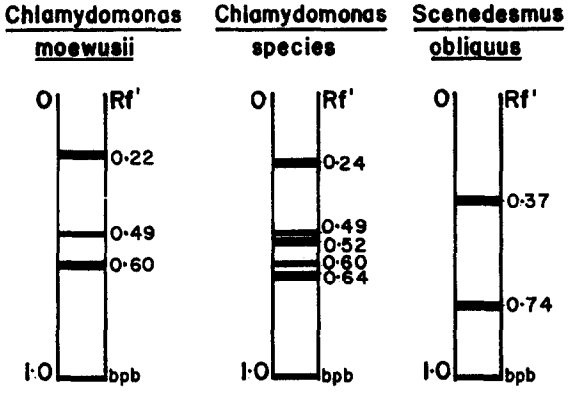

FIG. 4. HYDROGENASE BAND PATTERNS OF WHOLE CELL ADAPTED ALGAE.

Multiple forms of algal hydrogenases were obtained by the procedure outlined by Ackrell *et al.*[15] The position of each reduced dye band (R_f) was measured from the top of the gel and recorded as the ratio of this distance to the distance travelled by the marker dye bromophenol blue (bpb).

which are unique even in closely related species of algae. There is some indication that algal species within the same genus have certain hydrogenase bands in common. Two of the three bands obtained from *Chlamydomonas moewusii* have the same mobility as two of the bands found in *Chlamydomonas* sp.

There is no indication that during the adaptation process one of the hydrogenase isoenzymes precedes another in formation. Instead, at the earliest possible level of isoenzyme detection, all bands are detected. A shift to higher R_f values is noticed in cell-free extracts from dried cell preparations which have been stored at –20° under hydrogen for 5–15 days.

The passage of the hydrogenase into 7·5% polyacrylamide gel suggests that the algal enzymes are of moderate molecular weight (approximately 60,000). This is further substantiated by the facts that the R_f values for hydrogenase isoenzymes from algal preparations are similar to those of bacterial systems,[15] and by the appearance of the hydrogenases of *Chlamydomonas moewusii*, *Chlamydomonas* sp., and *Scenedesmus obliquus* in the supernatant fraction after centrifugation at 160,000 *g* for one hour. This is not necessarily a contradiction to the statement by Abeles,[9] that hydrogenase activity in *Chlamydomonas eugametos* is associated with particles sedimenting at 60,000 *g* after 1 hr, since the drying procedure

[15] B. A. C. ACKRELL, R. N. ASATO and H. F. MOWER, *J. Bact.* **92**, 282 (1966).

followed by sonication and centrifugation probably separates hydrogenase from any particle to which it may have been attached.

It is well known that the hydrogenases of algae are extremely susceptible to inactivation by oxygen.[2,9] Figure 5 shows the effect of air on dried cell suspensions of *Chlamydomonas moewusii* and *Chlamydomonas* sp. The samples were exposed to air for varying periods of time in Warburg flasks. Although significant loss in activity occurs after a short period of exposure to air, there is still measurable enzyme activity after 1 hr. Whole cell suspensions of newly adapted algae are completely inactivated after 15 min application of the same procedure. These preparations, however, were not always sufficiently stable in air to permit the 2–3-hr electrophoresis necessary to separate the hydrogenase isoenzymes. Work to stabilize these enzyme preparations further is now in progress.

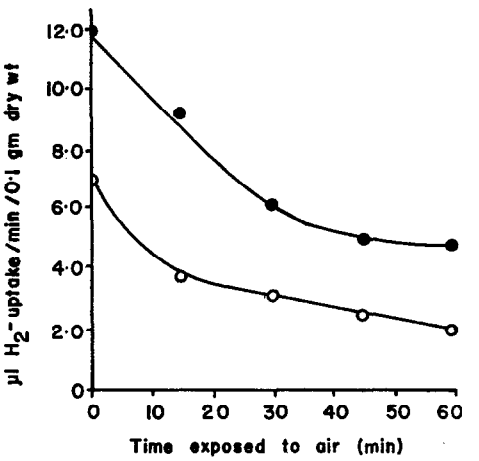

FIG. 5. EFFECT OF AIR ON HYDROGENASE ACTIVITY FROM DRIED CELL SUSPENSIONS.

Whole cell suspensions (1·0 g wet wt./ml) of *Chlamydomonas moewusii* and *Chlamydomonas* sp. were adapted 22 hr and dried *in vacuo*. The cells were anaerobically resuspended in 0·1 M phosphate buffer, pH 7·0, to 0·1 g dry wt./ml. 0·5 ml samples were assayed for hydrogenase activity following H_2-uptake with methylene blue. H_2 was used in the gas phase. As before, flasks without dye were used as controls.

●——●: *Chlamydomonas moewusii.*
○——○: *Chlamydomonas* sp.

MATERIALS AND METHODS

Cultures of *Chlamydomonas moewusii* (96), *Chlamydomonas intermedia* (222), *Chlamydomonas eugametos* (4, 5, and 6), *Gloeocapsa* sp. (LB-795), and *Nostoc muscorum* (456) were obtained from the Indiana University Algae Culture Collection. *Chlamydomonas pseudogloe* (12235) and *Scenedesmus obliquus* (11457) were obtained from the American Type Culture Collection; Rockville, Maryland. One species of *Chlamydomonas* (Baker A-1.4), isolated from Rogers Lake, Montana, was kindly supplied by Professor G. E. Baker, Department of Botany, University of Hawaii. *Chlorella pyrenoidosa* was supplied by Dr. G. A. Barber, Department of Biochemistry, Ohio State University. Samples of marine algae, *Ulva faciata*, and representative species of *Caulerpa, Dictyosphaeria, Hypnea, Sarggassum*, and *Turbinaria* were collected from the Waikiki Natatorium in Honolulu, Hawaii.

All the freshwater algal cultures were grown in modified Bristol's medium which contained per liter; $CaCl_2$—0·025 g, $NaNO_3$—0·25 g, $MgSO_4.7H_2O$—0·075 g, K_2HPO_4—0·075 g, KH_2PO_4—0·175 g, NaCl—0·025 g, and 40 ml of soil extract. Soil extract was prepared in the following manner: Equal portions of glass-distilled water and garden soil were combined and steamed for 1 hr on two consecutive days. After standing for 2 days, the supernatant solution was decanted and filtered. The filtrate was adjusted to pH 7·5 and sterilized to be used as needed. In addition to this medium, the cultures were made 10^{-4} M with $FeSO_4$ and 2×10^{-4} M with EDTA according to Sasaki.[16]

[16] H. SASAKI, *Plant Cell Physiol.* 7, 321 (1966).

The cells were grown in 1·0-l. Roux flasks, each containing approximately 750 ml of the medium, under continuous illumination from two 96 W fluorescent lamps. The cells were grown without shaking at 24°. A mixture of 95% air and 5% CO_2 was first passed through 1% Orange G (Matheson Company, Inc., East Rutherford, New Jersey) to reduce microbial contamination, and then continually bubbled through the algal cultures. Strict aseptic techniques were observed throughout the growth period. Algal samples were periodically checked for bacterial contamination by streaking the samples onto nutrient agar plates, both during growth and dark adaptation. Prior to adaptation, 1 part of concentrated "Zephiran Chloride" (Winthrop Laboratories, New York) was added to 500 parts of the algal suspension to further minimize bacterial contamination. No change in hydrogenase activity was observed as a result of this addition. When bacterial growth did occur, the contaminants were subcultured, and subsequently assayed for hydrogenase activity. At no time was hydrogenase activity observed in any contaminant.

Algae grown 3–7 days, were harvested by centrifugation (400 *g* for 10 min), washed three times with 0·1 M phosphate buffer, and resuspended in the same buffer to 1·0 g wet wt./ml. Except where specified, pH 7·0 phosphate buffer was used in all experiments. When larger quantities of cells were necessary, two 5-day-old 1·0-l. cultures were used to inoculate 40 l. These cultures were grown under the same conditions except that, due to the larger volume, the light intensity was somewhat less and cell yield per liter was less. The level of hydrogenase activity often varied considerably from culture to culture despite attempts to standardize growth conditions. Thalli of the marine algae were washed three times with distilled H_2O, blotted dry and weighed, cut into small segments, and suspended in the buffer solution to 1·0 g wet wt./ml. All algal suspensions were flushed with H_2 gas unless otherwise stated and allowed to adapt at 25° for the time periods shown in the experiments.

Hydrogenase activity was measured manometrically. Assays were carried out at 30° using either methylene blue and following the rate of hydrogen uptake, or using reduced methyl viologen and following the rate of hydrogen evolution. In some instances other redox dyes were used. These were benzyl viologen, 1-1′-tetramethylene-5-5′-dimethyl-2-2′ dipyridylium dibromide, 1-1′-tetramethylene-2-2′-bipyridylium-diiodide, and 1-1′-trimethylene-2-2′-dipyridylium-diiodide. Benzyl viologen and methyl viologen were obtained from Mann Research Laboratories. Methylene blue was supplied by Conway Products Co., New York. The dipyridylium dyes were obtained through the courtesy of Dr. E. A. Calderbank of I.C.I., Jealott's Hill Research Station, Bracknell, Berks., England, and Dr. R. C. Valentine of the University of California at Berkeley, California.

Cell-free extracts were prepared by drying the adapted cell suspensions *in vacuo* and grinding the residue into a powder. The ratio of dry weight to wet weight was 0·10 to 0·12 for species of *Chlamydomonas*. The powder was resuspended 1:10 (w/v) in phosphate buffer in the presence of dithionite, using methyl viologen as an indicator of reduction. 10 ml of the suspension was sonified 2 min at 100 W with a Branson Sonifier Cell Disrupter (Model W-185-C). Before suspension, the powder could be stored frozen under H_2 several days without appreciable loss of hydrogenase activity. After sonication, the suspension was centrifuged anaerobically at 160,000 *g* for 1 hr in a Beckman L2-65 Ultracentrifuge. The supernatant solution could be stored frozen under H_2 gas several days without appreciable loss in activity. Hydrogenase isoenzymes were detected by the polyacrylamide gel electrophoresis method of Ackrell *et al.*[15]

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