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ADAPTATION OF HYDROGENASE IN CELL-FREE PREPARATIONS FROM *CHLAMYDOMONAS**

M. A. WARD

Department of Botany, University of Hawaii, Honolulu, Hawaii

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Abstract—It has been demonstrated that hydrogenase activity can arise by adaptation in cell-free extracts of two *Chlamydomonas* species. The extract is obtained by sonication of a whole cell suspension followed by centrifugation at 160,000 *g* for 1 hr. The time course of adaptation is similar to that obtained from whole cells. The pH optima for both the cell-free adapted hydrogenase and the adaptation process is 7·0. Adaptation does not occur below 2·5°. Unadapted extracts may be stored for at least 4 days at 0·5–2·5° under hydrogen and then adapted at 25°. Adaptation of cell-free extracts is not inhibited by light. Three hydrogenase isoenzymes from adapted cell-free extracts of *Chlamydomonas* sp. are obtained as bands by electrophoresis on polyacrilamide gel. They are identical to three of the five isoenzyme bands obtained from extracts of adapted whole cells of this organism. One hydrogenase band is obtained from adapted cell-free extracts of *C. moewusii*. It is identical to one of the three isoenzyme bands obtained from adapted whole cell extracts of the same species. Cell-free adapted preparations are more stable to air than either adapted whole cells or dried cell preparations from adapted whole cells. ATP may exhibit a regulatory effect on the adaptation process. Adaptation does not occur until the extract is devoid of ATP.

INTRODUCTION

HYDROGENASE from algae adapted anaerobically in the dark was first observed by Gaffron[1] in 1940 from whole cells of *Scenedesmus*. Since then the enzymes have been found[2, 3] in many different algae from five taxonomic divisions. Work to elucidate further the roles of such enzymes in photosynthesis and related reactions has progressed slowly. Attempts to characterize the enzyme have proved unsatisfactory due to its extreme susceptibility to inactivation by oxygen, and the difficulties experienced in obtaining purified preparations.[4] Although cell-free preparations have been obtained from adapted whole cells, the activity has been found to be associated with particles of various sizes which render characterization studies difficult.[5, 6]

This report describes the adaptation of hydrogenase in cell-free preparations from two *Chlamydomonas* species. Several properties of these soluble hydrogenases are also described. These observations present further evidence that *de novo* synthesis of hydrogenase does not occur during adaptation. Comparisons of some of these properties are made with similar observations from adapted whole cells.

* 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] H. GAFFRON, *Am. J. Botany* **27**, 273 (1940).
[2] E. KESSLER, *Handb. Pflanzenphysiol.* **5**, 951 (1960).
[3] C. J. P. SPRUIT, *Physiology and Biochemistry of Algae*, p. 49, Academic Press, New York (1962).
[4] N. I. BISHOP, *Ann. Rev. Plant Physiol.* **17**, 185 (1966).
[5] F. B. ABELES, *Plant Physiol.* **39**, 169 (1964).
[6] Y. FUJITA and J. MYERS, *Plant Physiol. Suppl.* **39**, xii (1964).

RESULTS AND DISCUSSION

The adaptation time curves (Fig. 1) of hydrogen-adapted whole cells and cell-free extract from *Chlamydomonas moewusii* are similar with respect to their general shape. As in whole cell suspensions, hydrogenase activity in cell-free adapted extracts may be detected after short adaptation times, but this activity is considerably lower than the maximum activity attained at 23 hr. It may be that this low activity is endogenous to both whole cell and cell-free preparations and is independent of that which arises after longer adaptation times.

The maximum activity obtained from the cell-free preparations is higher than that found with whole cells. It is likely that degradation reactions competing with adaptation are less

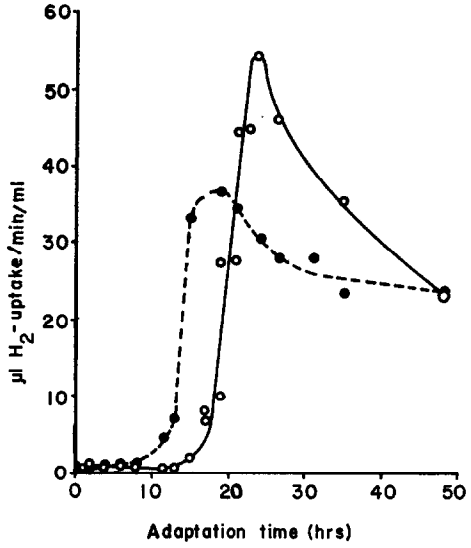

FIG. 1. ADAPTATION TIME CURVES FOR A CELL-FREE EXTRACT AND FOR WHOLE CELLS OF *Chlamydomonas moewusii*.

Hydrogenase activity was measured following hydrogen uptake with methylene blue. One side-arm of the Warburg flask contained 6·4 μmoles of methylene blue in 0·2 ml of 0·1 M phosphate buffer, pH 7·0. The other side-arm contained 0·5 ml of a 0·6 g wet wt./ml suspension of either adapted whole cells or cell-free extract. Cell-free extract was prepared by sonication of a whole cell suspension of 0·6 g wet wt./ml followed by centrifugation at 160,000 *g* for 1 hr. The supernatant was then adapted for varying time periods. The center well contained 0·1 ml of 14% NaOH to which was added 0·1 ml of 20% pyrogallol just prior to flushing. The total volume was made up to 3·2 ml with 0·1 M phosphate buffer, pH 7·0. The gas phase was H_2. A control, without dye, was used for each assay.

●——●: Adapted whole cells.
o——o: Adapted cell-free extract.

effective in cell-free extracts than in whole cells. This may be due to several factors. Any particulate material capable of inhibiting the adaptation process would not be found in cell-free extracts. Also, dilution of metabolites and enzymes involved in degradation may occur in cell-free extracts. Once rapid adaptation begins, the rate of hydrogenase activation is approximately the same for whole cells as it is for the cell-free system. An explanation for this rapid increase in the cell-free system appears later in the discussion. In general, the time required for maximum activity in cell-free extracts is longer than that required for whole cells. Since the actual adaptation times and activities vary from culture to culture of the same species, duplicate time-course experiments were performed.

The pH optimum of adapted cell-free hydrogenase, and the pH optimum of the adaptation process (Fig. 2) is 7·0. These optima are the same as that obtained with a cell-free pre-

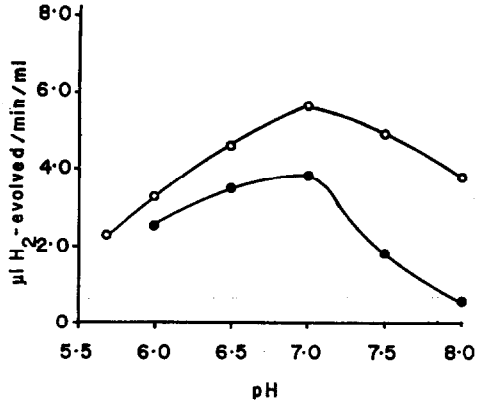

FIG. 2. COMPARISON BETWEEN THE pH PROFILE OF CELL-FREE ADAPTED HYDROGENASE ADAPTED AT pH 7·0, AND THE pH PROFILE OF THE CELL-FREE ADAPTATION PROCESS USING *Chlamydomonas* SP.

Hydrogenase assays were carried out by following H_2-evolution from reduced methyl viologen. 5·5 µmoles of methyl viologen reduced with 3 µmoles of sodium dithionite were used in the assay. 0·1 M phosphate buffers ranging in pH from 5·7–8·0 were used in both experiments. Controls, without reduced dye, were run with each assay. The gas phase was He.

o——o: pH profile of cell-free extract adapted at pH 7·0.
●——●: pH profile of cell-free extract adapted at various pH's and assayed at pH 7·0.

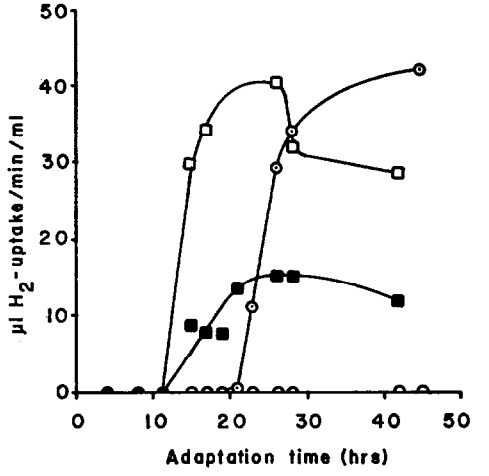

FIG. 3. EFFECT OF TEMPERATURE ON ADAPTATION.

Hydrogenase assays and controls were carried out as described under Fig. 1. The gas phase was hydrogen. Samples were adapted at the temperature ranges as described below.

●——●: Hydrogenase activities of all samples to 12 hr adaptation.
o——o: Hydrogenase activities of extracts adapted between 0·5–2·5°.
⊙——⊙: Hydrogenase activities of extracts adapted between 19·5–21·5°.
□——□: Hydrogenase activities of extracts adapted between 24–26°.
■——■: Hydrogenase activities of extracts adapted between 37–39°.

paration from adapted whole cells of *Chlamydomonas* sp.,[7] and are similar to the pH optimum of the adaptation process of whole cells.[7]

Temperature exhibits an effect on the adaptation of hydrogenase (Fig. 3) from cell-free preparations of *C. moewusii*. Over the temperature range of 0·5–2·5°, adaptation does not

[7] M. A. WARD, *Phytochem.* **9**, 259 (1970).

occur. However, extracts adapted within this temperature range, when brought up to 25°, adapt readily. The hydrogenase activity of such preparations is approximately 65 per cent of that obtained in preparations initially adapted at 25° for the same adaptation time. In addition, extracts stored at 0·5–2·5° and adapted at 25° are stable for at least 40 hr. Samples can be stored at 0·5–2·5° for at least 4 days without loss of adaptive ability. Samples stored under hydrogen at this temperature range exhibit a higher hydrogenase activity than comparable samples stored under air.

TABLE 1. EFFECT OF LIGHT UPON ADAPTATION OF CELL-FREE EXTRACTS FROM *Chlamydomonas moewusii*

Adaptation time (hr)	Activity (μl H_2 uptake/min/ml)	
	Control (dark adapted)	Light
13·5	5·14	5·14
16·3	51·4	28·0
19·7	54·0	52·6
36·0	44·5	52·1

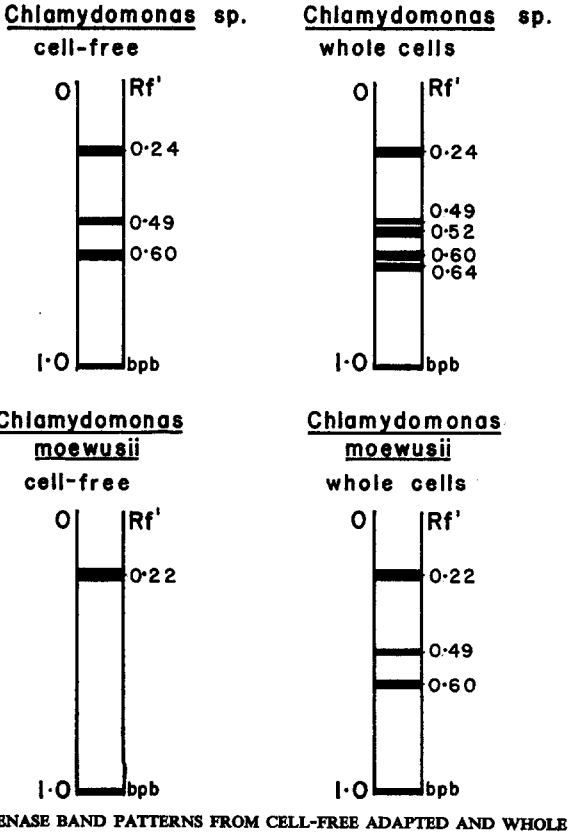

FIG. 4. HYDROGENASE BAND PATTERNS FROM CELL-FREE ADAPTED AND WHOLE CELL ADAPTED PREPARATIONS.

The multiple forms of algal hydrogenases were obtained by the procedure outlined by Ackrell *et al.*[11] 0·1 ml of the extracts were placed on each gel column. bpb is bromophenol blue marker.

Above 21° more rapid initial adaptation occurs and maximum activity is achieved more rapidly. The adaptation time at which activity is first observed in extracts adapted at 37–39° is the same as that obtained for extracts adapted between 25–26°. Also, the maximum activity is reached in the same time. However, the maximum activity obtained in extracts adapted at 37–39° is considerably lower than that of extracts adapted at 25–26°. The low initial activity previously observed in cell-free preparations (between 0 and 10 hr adaptation) could not be detected in the samples used for this experiment.

Gaffron[1] has observed that adaptation of hydrogenase in whole cell suspensions of algae does not proceed in the light. This observation has been confirmed by several workers.[8–10] Gaffron deduced that this was due to the continued formation of oxygen

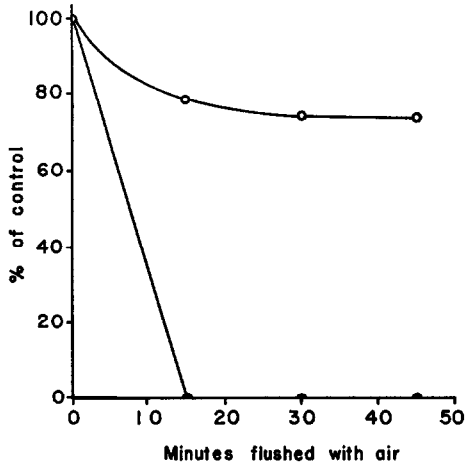

FIG. 5. EFFECT OF AIR ON HYDROGENASE ACTIVITY FROM CELL-FREE ADAPTED EXTRACTS AND WHOLE CELLS OF *Chlamydomonas moewusii*.

Adaptation time was 18 hr. Warburg flasks containing the enzyme were flushed with air for the time periods shown. The air was replaced with hydrogen by flushing for 10 min. Hydrogenase activity was determined as described under Fig. 1.

o——o: Cell-free adapted extracts.
●——●: Adapted whole cells.

produced through the photolysis of water during photosynthesis. In view of this observation, it is interesting to note that hydrogenase from cell-free extracts can be adapted in the presence of light (Table 1). Since the oxygen-evolving mechanism is absent in the cell-free extracts, this result lends support to Gaffron's theory of light inhibition of adaptation in whole cells.

Figure 4 shows the relationship between hydrogenase isoenzymes obtained from adapted whole cells and adapted cell-free extracts of *Chlamydomonas* sp. and *C. moewusii*. Efforts to resolve unadapted cell-free extracts by gel electrophoresis and then adapt the enzymes directly on the gel were not successful. With both algae, there are isoenzyme bands common to the cell-free adapted preparations and adapted whole cells. The number of isoenzymes obtained

[8] H. HARTMAN and A. I. KRASNA, *J. Biol. Chem.* **238**, 749 (1963).
[9] R. A. GOLDSBY, *Lawrence Radiation Lab.* Berkeley, Calif. UC-4 Chemistry, 1 (1961).
[10] G. GINGRAS, R. A. GOLDSBY and M. CALVIN, *Arch. Biochem. Biophys.* **100**, 178 (1963).

from adapted cell-free extracts is less than that derived from whole cells. The reasons for this are not presently known. The number of isoenzymes is not directly related to the total hydrogenase activity of a given preparation. The cell-free adapted extracts reach a higher total activity than whole cells but have fewer isoenzyme forms. This is in agreement with the findings of Ackrell *et al.*[11] on multiple hydrogenase forms from bacteria. There is no evidence that the number of isoenzymes varies with adaptation time. Rather, as is the case with whole cells, all the forms appear simultaneously at any adaptation time in which activity can be measured.

The active hydrogenase from adapted cell-free extracts of *C. moewusii* is much more stable to air (Fig. 5) than the hydrogenase in adapted whole cells which loses 100 per cent of its activity after 15 min. Even after 45 min exposure to air, 75 per cent of the activity remains.

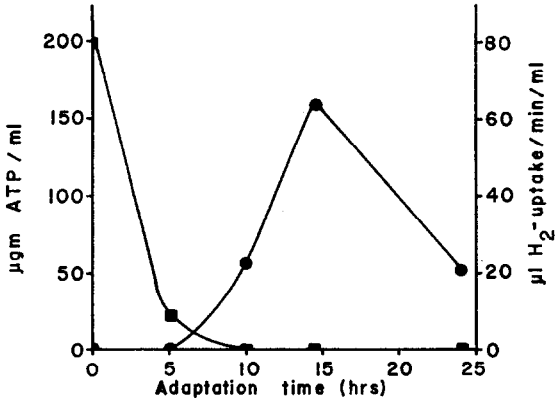

FIG. 6. EFFECT OF ENDOGENOUS ATP ON HYDROGENASE ADAPTATION IN CELL-FREE EXTRACTS OF *Chlamydomonas moewusii*.

ATP assays were carried out as described in the text. Hydrogenase activity at the various adaptation times was measured as described under Fig. 1.

●——●: Hydrogenase activity.
■——■: ATP content.

It is also considerably more stable to air than hydrogenase from dried cell suspensions of adapted whole cells of the same organism,[7] which loses 58 per cent of its activity after 45 min exposure to air. Moreover, no significant loss in enzyme activity is found between 48 and 92 hr adaptation in cell-free adapted preparations. This may be considered further evidence for the increased stability to air of cell-free adapted hydrogenase.

Adaptation of cell-free extracts can be achieved in the presence of 1×10^{-3} M arsenate. With 2×10^{-3} iodoacetate adaptation is completely inhibited. The same concentrations of bisulfite and sodium fluoride inhibit adaptation to a greater extent in cell-free extracts than in whole cells.[7] Presumably this is due to the permeability barriers present in whole cells.

Dialysis of cell-free extracts against 5×10^{-3} M phosphate buffer, pH 6·8, results in 75 per cent inhibition of adaptation. Passing the extract through a DEAE-cellulose column at 4° inhibits adaptation as much as 67 per cent. This observation may suggest ferredoxin as a requirement for adaptation. As noted with whole cells,[7] cell-free extracts can be adapted under carbon monoxide.

[11] B. A. C. ACKRELL, R. N. ASATO and H. F. MOWER, *J. Bacteriol.* **92**, 828 (1966).

In a preliminary experiment, the ATP-generating system of Burns[12] was added to an unadapted cell-free extract of *C. moewusii*. This addition results in complete inhibition of adaptation for at least 40 hr. The components of the system were added separately to a pre-adapted extract with no effect. Figures 6 and 7 show, respectively, the effect of endogenous and added ATP on the adaptation process. Addition of ATP delays adaptation. In both experiments hydrogenase activity is not detected until ATP is no longer present in the extract. The maximum level of hydrogenase activity reached is not dependent upon ATP concentration.

It may be postulated that ATP functions in a regulatory capacity in that the disappearance of ATP is requisite to the rapid increase in hydrogenase activity (Fig. 1). One would also suspect that ATP disappears more rapidly at higher temperatures causing a more rapid appearance of hydrogenase activity (Fig. 3). In contrast, the fact that no adaptation occurs

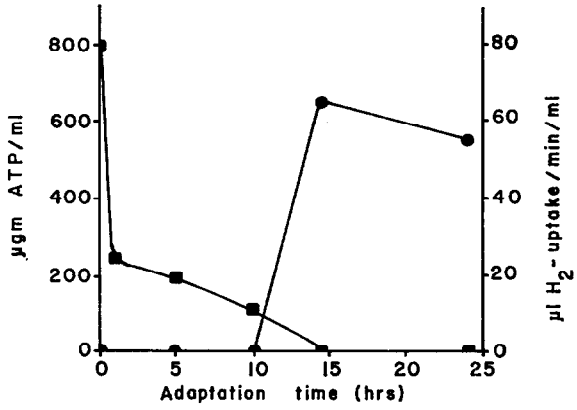

FIG. 7. EFFECT OF ADDED ATP ON HYDROGENASE ADAPTATION IN CELL-FREE EXTRACTS OF *Chlamydomonas moewusii*.

The extract at zero time adaptation was made 10^{-3} M with ATP in addition to the endogenous amount. The amount of ATP shown at zero time adaptation is calculated from the amount of endogenous ATP at time zero (the same as in Fig. 6 at time zero). All other points determined by direct assay of the extract. Hydrogenase activity was determined as described under Fig. 1.

●——●: Hydrogenase activity.
■——■: ATP content.

at low temperatures may be due to the persistent presence of ATP. The relative rapidity with which added and endogenous ATP initially disappears suggests the presence of ATPase activity.

MATERIALS AND METHODS

A culture of *Chlamydomonas moewusii*[6] was obtained from the Indiana University Algae Culture Collection. A stock culture of *Chlamydomonas* sp. (Baker A-1.4), isolated from Rogers Lake, Montana, was supplied by G. E. Baker, Department of Botany, University of Hawaii.

The algal cultures were grown and cell suspensions were prepared as described previously.[7] Except where otherwise stated, the cells were suspended 0·6 g wet wt./ml in 0·1 M phosphate buffer, pH 7·0. Cell-free preparations were prepared by sonication of 50 ml of the cell suspension for 2 min at 100 W with a Branson Cell Disrupter (Model W-185-C). The sonicate was then centrifuged at 160,000 *g* for 1 hr in a Beckman L2-65 Ultracentrifuge. The supernatant solution served as the cell-free extract. Prior to adaptation, 1 vol. conc. "Zephiran Chloride" solution (Winthrop Laboratories, New York) was added to 750 vol. of extract to

[12] A. SAN PIETRO, *Non-Heme Iron Proteins*, p. 289, Antioch Press, Yellow Springs, Ohio (1965).

decrease the probability of bacterial contamination. Concentrations of "Zephiran Chloride" above this level caused protein denaturation in the extracts and resulted in inhibition of adaptation. Microscopic examination revealed no bacterial contaminants before or after adaptation. The protein concentration of the supernatant solution was determined by the method of Gornall *et al.*[13] Protein concentration was consistently between 11 and 13 mg/ml.

Adaptation of extracts for time periods less than 1 hr was carried out directly in Warburg flasks. Each flask was allowed to equilibrate for at least 5 min. Adaptations at times longer than 1 hr were equilibrated for 15 min. Extracts were adapted in 40-ml serum bottles containing 10 ml of extracts. After placing the extracts under H_2, the bottles were wrapped in aluminum foil and the extracts were allowed to adapt. Adaptation in the light was done by illuminating the extract with a 250-W flood lamp 2 ft from the sample. An aquarium tank filled with water was placed between the light and the sample to prevent heating. Samples were withdrawn anaerobically with syringes after various adaptation times and assayed for hydrogenase activity. The gas phase in all adaptations was H_2 at atmospheric pressure unless otherwise specified. All samples were adapted at 25° unless otherwise specified.

Hydrogenase activity was determined manometrically by measuring H_2 uptake via methylene blue (Conway Products Co., New York), or by measuring H_2 evolution with reduced methyl viologen (Mann Research Laboratories). Hydrogenase assays were run at 30°.

The ATP content of adapted extracts was determined spectrophotmetrically by the method of Kornberg[14]. Commercial ATP assay kits and ATP were obtained from Calbiochem, Los Angeles, California. The adapted extracts were heated for 10 min at 80° and centrifuged to remove interfering protein. ATP was not affected by this heating procedure.

The stability of the adapted hydrogenase to air was determined by flushing Warburg flasks containing the enzyme with air. The flasks were flushed for varying periods of time after which the gas phase was replaced with H_2 and allowed to equilibrate for 10–15 min. The enzyme extract was then assayed for hydrogenase activity and compared with the appropriate control.

Hydrogenase isoenzymes were demonstrated by the polyacrilamide gel electrophoresis method of Ackrell *et al.*[11]

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[13] A. G. GORNALL, C. S. BARDAWILL and M. M. DAVID, *J. Biol. Chem.* **177**, 751 (1949).
[14] A. KORNBERG, *Methods in Enzymology*, Vol. 2, p. 497, Academic Press, New York (1955).